Vol. XLII, No. 3 (June 1961), was issued on 16.6.61.

THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XLII AUGUST, 1961 NO. 4

THE EFFECT OF THE COMPONENTS OF ADJUVANT EMULSIONS ON LYMPHATICS IN THE MOUSE'S EAR

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Received for publication August 6, 1960

In a previous paper it was shown that Freund's adjuvants stimulated proliferation of lymphatic vessels at the site of injection in the ears of mice (Dale, 1960). The first part of the present study was undertaken to investigate whether this effect was due to one or more particular constituents of the adjuvant emulsion or whether it was a function of their acting in combination. The object of the second part of the study was to determine the effect on lymphatic vessels of other oil and water emulsions which have been tested as adjuvants in sensitization procedures. Several oil-and-water emulsions which have been tested for their effect in enhancing antibody production in rabbits have been found to be ineffective or only partially effective (Freund, 1948). It had been suggested in a previous paper (Dale, 1960) that one of the many possible factors involved in the increased antibody production with an effective adjuvant emulsion (Bayol F + Arlacel A + Saline) might be the proliferation of lymphatic vessels at the site of injection with the consequent possibility of " channelling " of antigen to local lymph glands. It was considered that it might be of interest to determine the effect on lymphatics of oil and water emulsions which were unsuccessful in enhancing antibody production as compared with the effect of adjuvants known to potentiate antibody production.

MATERIALS AND METHODS

The animals used were adult female albino mice approximately three months old.

Experiment 1

For experiment 1a, 70 mice were randomly allocated to 7 treatment groups. For experiment 1b, 55 mice were allocated to 9 treatment groups. The preparations used for injection of the ears are set out in Table I. In experiment 1a the 4 components of Freund's adjuvants emulsion were tested separately and in various combinations on the mouse's ear, but in each case the total volume of fluid injected into the ear was the same, *i.e.*, 0·005 ml. In experiment 1b the preparations were injected in the quantities in which they occur in the total adjuvant emulsion.

Experiment 2

Expt. 2a: Twenty-one mice were allocated to 2 treatment groups and the ears injected as follows:

Group BAS (10 mice): Bayol F + Arlacel A + Saline (2 : 1 : 2).
Group AcAS (11 mice): Arachis Oil + Arlacel A + Saline (2 : 1 : 2).

Expt. 2c: Eighteen mice were allocated to 2 groups and the ears injected as follows:
Group BAS (9 mice): Bayol F + Arlacel A + Saline (2 : 1 : 2).
Group BTwS (9 mice): Bayol F + Tween 80 + Saline (2 : 1 : 2).

Expts. 2b and 2d were repetitions of 2a and 2c respectively, done at a later date with a different batch of mice and with 6 animals in each group. The volume of emulsion injected in each case was 0·005 ml. (Expts. 1b, and 2b and 2d, were done concurrently, mice of the same batch being used, so that Group BAS is common to each of these experiments.)

The emulsions were made in a pestle and mortar, the oil and the saline being added alternately drop by drop to the emulsifying agent. The resulting preparation was then further emulsified in a syringe, by repeatedly forcing it through a needle of small bore.

The mice were anaesthetized with ether and 0·005 ml. of the requisite preparation was injected subcutaneously in the centre of the right ear with an Agla micrometer syringe and a No. 20 record needle. The left ear served as an uninjected control in each animal. After injection the mice were left for varying periods of time (1–4 months in expt. 1a, 2–5 weeks in expts. 1b and 2) and their ears were then examined for evidence of lymphatic proliferation. The methods of injection and photographic recording were described previously (Dale, 1960).

Assessment of degree of proliferation of lymphatics

Photographs of the injected ears, printed in as uniform a manner as possible, were used in assessing the degree of proliferation of the lymphatics. For the overall comparison of groups, as no accurate quantitative measure of proliferation was feasible, it was decided to choose 4 arbitrary reference photographs of increasing complexity of lymphatic pattern, and then to grade all the photographs into 5 classes, (I, II, III, IV, and V) with reference to these arbitrary standards. In assessing the amount of proliferation, both density of meshwork and the area involved were taken into consideration. Fig. 1B, C, D, and E represent the lower boundaries of Classes II, III, IV and V respectively. (Fig. 1A is included to show an example of Class I, and 1F to show an extreme example of Class V).

The grading and ranking of the photographs was done separately by 2 independent observers who, at the time of grading did not know the treatments given. Initially there were disagreements on 14 out of 177 photographs, and these were then resolved by joint discussion.

TABLE I.—*Volumes of Substances Injected Subcutaneously into the Right Ears of Mice in Experiments 1a and 1b*

	Experiment 1a				Experiment 1b			
Group	Bayol F (ml.)	Arlacel A (ml.)	Saline (ml.)	Total volume (ml.)	Bayol F (ml.)	Arlacel A (ml.)	Saline (ml.)	Total volume (ml.)
BAST	—	—	—	—	0·002	0·001	0·002	0·005
BAS	0·002	0·001	0·002	0·005	0·002	0·001	0·002	0·005
BT	0·005	—	—	0·005	0·002	—	—	0·002
AT	—	0·005	—	0·005	—	0·001	—	0·001
ST	—	—	0·005	0·005	—	—	0·002	0·002
B	0·005	—	—	0·005	0·002	—	—	0·002
A	—	0·005	—	0·005	—	0·001	—	0·001
S	—	—	0·005	0·005	—	—	0·002	0·002
BA	—	—	—	—	0·002	0·001	—	0·003

The groups have been designated with the initials of the preparations injected:
B = Bayol F. A = Arlacel A. S = Saline.

In all groups containing the letter T, 1 mg. of a preparation of killed *Myco. tuberculosis* was added per ml. of injection fluid.

Statistical Analysis of Results

It was obvious that if the significance of differences between groups was to be assessed, a non-parametric test would be necessary. It was decided that the Mann-Whitney U-Test (two-tailed) would be used and the differences between groups would be accepted as significant at the level $P < 0{\cdot}05$. In some cases, the photographs of the 2 groups being compared were ranked in order of degree of proliferation, from least to greatest, without reference to the standard photographs, and a Mann-Whitney U test done on the basis of this ranking. In most instances however, it was not necessary to do this and the results of the grading of ears into 5 classes was used and all ears allocated to the same class were regarded as " ties " and ranked as such.

RESULTS

Experiment 1

As can be seen from Table II there was an increasing degree of proliferation of lymphatics in the various groups as follows, (" < " indicating a statistically significant difference) :

Expt. 1a : S, ST < A < AT < B, BAS.
Expt. 1b : S, ST < A < AT, BT < BA, BAS, BAST.

In expt. 1b, group B falls midway between A and AT and is not significantly different from either. In expt. 1a, it was not possible to classify the ears of the mice in group BT as there was too much tissue reaction and damage, ranging from oedema to perforation.

Thus the overall effects were as follows : (a) Saline, and saline + killed *Mycobacterium tuberculosis* (" Tb ") caused no lymphatic proliferation. (b) Arlacel A caused a slight amount of proliferation, which was increased when " Tb " was added to the Arlacel A. (c) Mixtures of Bayol F + Arlacel A, Bayol F + Arlacel A + Saline, and Bayol F + Arlacel A + Saline + " Tb ", all caused marked proliferation. (d) Bayol F alone caused mild proliferation in 0·002 ml. quantities and marked proliferation in 0.005 ml. quantities. Bayol F + " Tb " caused moderate proliferation in 0·002 ml. quantities and produced tissue damage in 0·005 ml. quantities.

Experiment 2 (see Table II)

When the photographs of the mice from expt. 2a were ranked according to the allocation to classes in Table II, the difference between the 2 groups was significant, and the same holds for expt. 2b.

When the ears of the 12 mice from expt. 2c were compared, after having been ranked according to degree of lymphatic proliferation, independently of the allocations in Table II, the difference between the 2 groups was significant, and the same holds for expt. 2d.

Thus in these experiments an emulsion of Bayol F, Arlacel A and Saline produced significantly more proliferation of lymphatics in the mouse's ear than either an emulsion of Bayol F, Tween 80 and Saline or an emulsion of Arachis Oil, Arlacel A and Saline.

DISCUSSION

Adjuvants are known to produce a local granuloma, and proliferation of lymphatics might be expected as part of the process of granuloma formation. It is possible that the proliferation of lymphatics with adjuvants is merely secondary

TABLE II.—*Degree of Lymphatic Vessel Proliferation in Ears of Mice Receiving Different Treatments*

Expt. No.	Group	Total number of mice	Class (Degree of proliferation) I	II	III	IV	V
1a	S	11	11	—	—	—	—
	ST	11	11	—	—	—	—
	A	9	4	3	1	1	—
	AT	9	1	2	—	6	—
	BAS	10	—	—	3	3	4
	B	9	—	—	—	6	3
1b	S	6	6	—	—	—	—
	ST	6	6	—	—	—	—
	A	6	2	3	1	—	—
	B	6	1	1	1	2	1
	AT	6	—	1	3	2	—
	BT	6	1	—	4	1	—
	BA	7	—	—	1	1	5
	BAST	6	—	—	—	2	4
	BAS	6	—	—	—	—	6
2a	BAS	10	—	—	3	3	4
	AcAs	11	2	4	2	2	1
2b	BAS	6	—	—	—	—	6
	AcAs	6	—	—	4	1	1
2c	BAS	9	—	—	1	2	6
	BTwS	9	3	1	3	1	1
2d	BAS	6	—	—	—	—	6
	BTwS	6	—	—	—	3	3

B = Bayol F. A = Arlacel A. S = Saline. T = Killed tubercle bacilli.
Ac = Arachis Oil. Tw = Tween 80.

Class I = No proliferation. Class V = Marked proliferation. See Fig.

to, or part of, the general tissue response to the continued presence of irritant substances. Coffin (1906) reported the growth of lymphatics in granulation tissue. On the other hand it might be possible that some preparations have a fairly specific effect in inducing proliferation of lymphatics. Work on acute inflammation has dissected out the chemical mediators responsible for various aspects of the inflammatory response, and it seems apparent that some substances have a fairly specific effect in increasing capillary permeability, others in causing vasodilation, etc. It may be the case that some substances, endogenous or exogenous, have a

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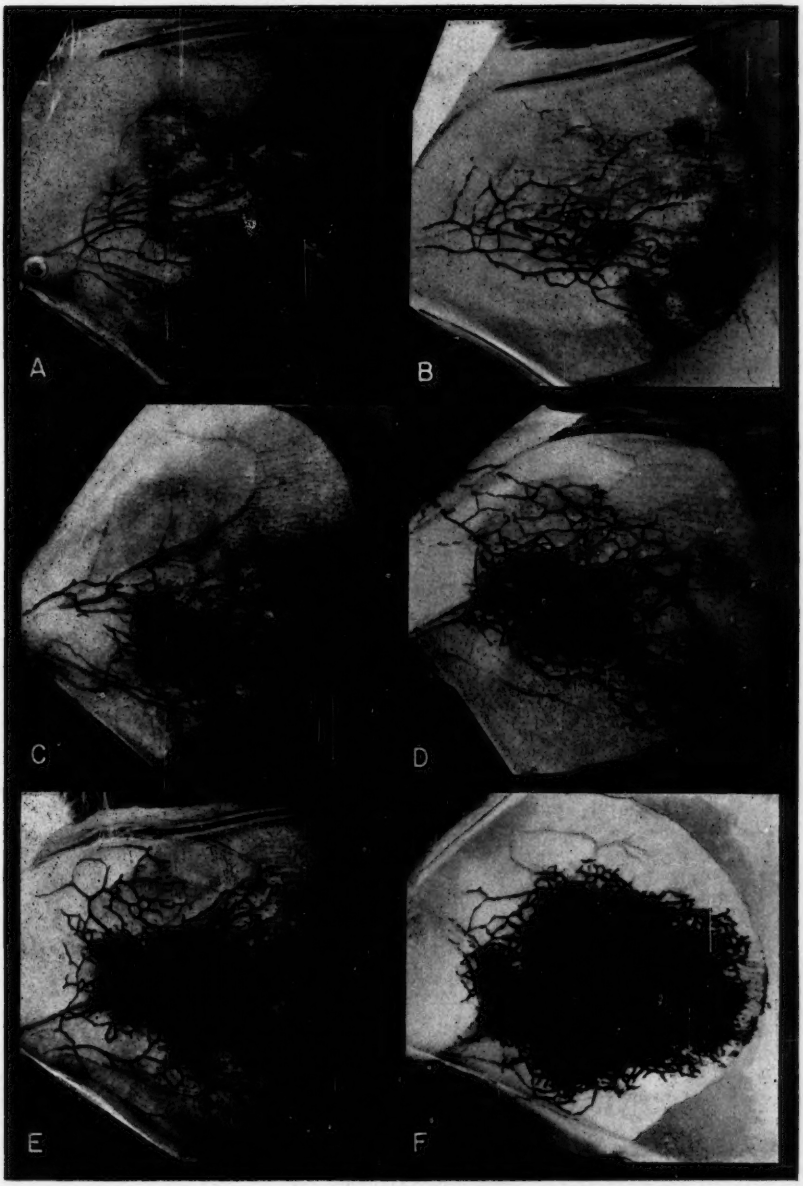
EXPLANATION OF PLATE

Photographs of mouse ears after injection of the lymphatics with Indian ink. B, C, D and E were used for " Standard Reference Points " in grading the degree of lymphatic proliferation.

A. Example of Class I.
B. Lower boundary of Class II.
C. Lower boundary of Class III.
D. Lower boundary of Class IV.
E. Lower boundary of Class V.
F. Example of marked degree of proliferation in Class V.



Dale.

relatively specific effect in inducing lymphatic vessel proliferation without markedly affecting other tissue components. In some of the experiments described above, an increase in the meshwork of lymphatic vessels was seen when there appeared to be relatively little macroscopic evidence of granuloma formation in the ear.

In antibody production and sensitization phenomena, the paraffin oil and the killed tubercle bacilli appear to be important both individually and together. Antibody production is enhanced when the mycobacterium fraction is not used and the antigen is incorporated into plain water-in-oil emulsion prepared with paraffin oil (Freund, 1948; Halbert, Mudd, and Smolens, 1946). But several studies have shown that killed tubercle bacilli or preparations of tubercle bacillary wax further increase the potentiating effect of water-in-oil emulsions on antibody production (Freund, 1948; Fischel, Kabat, Stoerk and Bezer, 1952; White, Coons and Connolly, 1955), while in the production of allergic encephalitis and aspermatogenesis, the presence of killed mycobacteria appears to be critical, and injections of emulsions of tissue suspensions with paraffin oil, but without killed mycobacteria, are not effective (Lipton and Freund, 1953; Freund, Lipton and Thompson, 1953; Freund, 1957). Raffel, Arnaud, Dukes and Huang (1949), however, showed that a wax fraction of the tubercle bacillus, though potentiating antibody production when incorporated into a water-in-oil emulsion, did not do so when injected as a suspension in water.

In considering the proliferative response of the lymphatics to the adjuvant mixture, the question arises here also, as to whether any particular component might have a more marked effect than others. In the present study both Bayol F and the emulsifying agent Arlacel A stimulated lymphatic vessel proliferation, and in general the effect was further enhanced by combining them or by adding killed tubercle bacilli to either. The suspension of killed bacilli alone, however, had no effect on the lymphatic vessels, and adding killed tubercle bacilli to the full water-in-oil emulsion did not further increase the already marked proliferative response to the latter.

It seems likely the overall effect on lymphatic vessels of the adjuvants, in the usual proportions, is not due to one particular constituent but is a result of their acting in combination.

It is of interest that when the individual constituents were injected in equal amounts as in experiment 1a, Bayol F used alone produced a very marked proliferation of lymphatics, as much in fact as the total adjuvant mixture. When the same quantity of Bayol F was given, combined with killed tubercle bacilli, there was marked tissue damage.

In the second part of the experiment the effect on lymphatics of two other emulsions was compared with that of Bayol F + Arlacel A + Saline. In a study of the effect of adjuvants on antibody formation, Freund (1948) tested various oil and water emulsions. He found that Bayol F and saline emulsions made with Tween 80, and Arachis Oil and saline emulsions made with Arlacel A, were ineffective in enhancing antibody production, whereas Bayol F and saline emulsions made with Arlacel A markedly potentiated formation of antibodies. It is of interest that the 2 emulsions quoted above as being ineffective as adjuvants in immunological procedures, had in the present study significantly less effect also in the production of lymphatic proliferation, than a Bayol F + Arlacel A + Saline emulsion.

This raises the possibility that there may in fact be some relationship between the stimulation of lymphatic vessel proliferation caused by Freund's adjuvants and their potentiating effect on antibody production.

SUMMARY

The constituents of Freund's adjuvants have been tested singly and in various combinations for their effect in stimulating lymphatic vessel proliferation in the mouse's ear. Saline, and saline with killed *Myco. tuberculosis* had no effect. Both Arlacel A and Bayol F, used alone, stimulated proliferation and in general the effect was increased if killed tubercle bacilli were added to either, and further increased if the two were combined. Marked proliferation was produced with the full water-in-oil emulsion both with and without killed tubercle bacilli. It is concluded that the overall effect on lymphatic vessels of the adjuvants is probably a result of their acting in combination and is not due to a particular constituent.

Two oil and water emulsions, Bayol F + Tween 80 + Saline, and Arachis Oil + Arlacel A + Saline, found by Freund (1948) to be less effective in enhancing antibody production than Bayol F + Arlacel A + Saline, were found to be less effective also in stimulating lymphatic vessel proliferation.

I wish to thank Professor T. Gillman for his encouragement and Dr. M. K. S. Hathorn for assistance. I am indebted to the Council for Scientific and Industrial Research for financial support. My thanks are also due to Mr. S. Archary and Mr. S. Rangiah for technical assistance, and to Mr. R. Kirk for photographic work.

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EFFECT OF X-IRRADIATION UPON THE DEVELOPMENT OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS IN GUINEA-PIGS

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Received for publication January 16, 1961.

WHILE many features remain unclear, it is generally accepted that encephalitis in guinea-pigs consequent upon injection of brain together with adjuvants is " allergic " in nature. From the pioneer work of Benjamin and Sluka (1908) and the literature reviewed by Taliaferro (1957), it would seem that antibody formation is reduced when whole body X-irradiation is carried out 12–24 hr. before antigen inoculation. Such treatment might be expected, therefore, to reduce the incidence of experimental allergic encephalitis (EAE). This expected effect was not encountered in practice, though this cannot be urged against the " allergic " nature of experimental encephalitis as has been suggested by Allegranza (1959).

MATERIALS AND METHODS

Preliminary experiments were carried out to determine the dose of whole body radiation which adult guinea-pigs (350–450 g.) would tolerate. Irradiation was carried out in a cardboard box divided into pens so that 6 animals at a time could be treated. The following details were kindly supplied by Dr. F. T. Farmer. " Kilovoltage 250 Kv. Filter 0·5 mm. Cu plus 1 mm. Al. H.V.L. 2 mm. Cu. Distance 150 cm. Dose Rate (in am.) 10·7 r/min. Allowing 30 per cent absorption in half the animal thickness, the average dose rate may be taken as 10·4 r/min." All irradiations were carried out as single exposures. In 2 animals from each batch of 6 irradiated as a group, blood counts were made before and some days after irradiation. A leucopenia of 30–50 per cent was commonly found and taken to indicate that treatment had been satisfactory.

A dose of 900 r proved uniformly fatal within 7–9 days. Of 8 animals dosed with 450 r, 5 died in 8–13 days and 3 survived. Of 12 animals exposed to 300 r, 6 survived. No animal exposed to 150 r succumbed. It was, therefore, decided to use 150 r as a standard dose.

Twenty-nine animals were submitted to 150 r whole body irradiation and 18 hr. later were injected intracutaneously in the dorsum of the right foot with 0·2 ml. of human white matter-adjuvant mixture made up of 4 parts human white matter, 3 parts Arlacel A, and 4 parts Bayol F with heat killed and dried tubercle bacilli. Each inoculum contained the equivalent of 15 mg. wet brain and 1 mg. tubercle bacilli. The particular mixture used was known to have only moderate pathogenicity. Animals were examined and their weights recorded daily, observations being continued for 7 weeks. Histological examination of brain, medulla, spinal cord (cervical and lumbar regions) as well as lymphatic system was carried out. The scoring system suggested by Kies and Alvord (1957) was adopted as affording some measure of the severity of the experimental disease.

In addition 32 control animals injected with the same antigenic mixture were available for comparison.

Skin sensitivity to the original white matter was assessed at 16 and again at 28 days after the injection and compared with that developed by non-irradiated animals. A volume of 0·1 ml. of 1/100 sterile suspension of white matter, centrifuged at 2000 r.p.m. for 1 min., was injected intradermally into a point in the depilated skin of the flank, whose thickness

as a pinched-up fold was first measured with a vernier-gauge. After 24 hr. and again at 48 hr. the diameter of the area of redness and the skin thickness was measured. The increase in thickness of the double fold of skin was expressed as a percentage of the initial thickness. In making these measurements care was taken to make them as comparable as possible by accurately centring the tissue to be measured between the jaws of the gauge. In this way some quantitative assessment of skin sensitivity could be made for comparative purposes. The diameter of red areola and presence or otherwise of central necrosis was also recorded.

RESULTS

Details of results are set out in the Table.

TABLE

Experimental animals				Controls			
		Skin sensitivity				Skin sensitivity	
No.	Disease index	16 days	28 days	No.	Disease index	16 days	28 days
112	3	4·3	17·7	114	1	28·2	40·9
119	3	—	—	115	1	29.0	50.0
120	3	10·4	50·7	116	0	42·5	53·1
134	+5	19·2	22·2	117	3	58·8	28·1
135	+5	36·4	35·6	118	3	67·1	50·7
139	+5	48·9	—	136	0	40·7	34·2
140	5	—	—	137	4	53·8	52·8
141	3	43·6	30·7	138	0	45·8	72·4
144	4	54·2	37·4	142	+5	86·7	—
145	+5	27·5	—	143	+5	67·3	—
146	3	54·2	62·5	147	3	30·2	64·2
149	+5	63·7	—	148	4	47·1	63·1
150	0	24·7	52·6	151	2	82·0	42·7
423	2	62·3	—	152	0	50·0	50·4
424	5	—	—	182	2	—	—
425	2	60·7	—	183	2	—	—
427	+5	33·3	—	184	1	—	—
428	+5	—	—	185	3	—	—
429	+5	—	—	186	3	—	—
430	5	—	—	187	4	—	—
431	1	68·4	—	188	0	—	—
432	4	20·8	—	189	4	—	—
433	+5	—	—	190	4	—	—
434	4	42·7	—	191	4	—	—
435	0	68·5	—	454	1	—	—
436	2	53·9	—	455	0	—	—
437	+5	—	—	456	0	—	—
438	2	66·6	—	457	0	—	—
440	4	36·0	—	458	0	—	—
				459	1	—	—
				460	0	—	—
				461	1	—	—

\+ = Died.
— = No test carried out.
Disease index = 0–5 according to the system of Kies and Alvord (1957).
Skin sensitivity expressed as per cent increase in thickness of skin at 24 hr.

Mortality

On the basis of 10 out of 29 experimental animals succumbing to EAE, whilst 2 out of 32 controls died, the χ^2 test (with Yates' Correction) shows $P = 0{\cdot}02 - 0{\cdot}01$ —a significant result.

Morbidity

On the basis of 27 out of 29 experimental animals falling ill with EAE as against 10 out of 32 controls, the χ^2 test (with Yates' Correction) shows $P = 0{\cdot}05 - 0{\cdot}02$ —a significant result.

Severity of disease

If the animals which died spontaneously are excluded and the influence of irradiation upon the remaining 19 considered from the point of view of their disease index scoring, the *t*-test shows $P_{37df} = 0{\cdot}05$–$0{\cdot}02$, *i.e.* the irradiation has increased the intensity of the disease process in those animals which did not actually die.

Skin sensitivity

Analysis of the measurements made showed that there was no significant reduction in skin sensitivity as a result of the irradiation either at 16 days ($P = 0{\cdot}2 - 0{\cdot}1$) or at 28 days ($P = 0{\cdot}5 - 0{\cdot}3$).

DISCUSSION

Maximal inhibition of antibody response is found when whole body X-irradiation precedes antigen inoculation by 12–24 hr. (Taliaferro, 1957). The present experiments, therefore, where antigen was given 18 hr. after irradiation, would appear to offer suitable conditions for any reduction in immune response to manifest itself.

Contrary to what was expected, however, such treatment enhanced the severity of EAE both as regards incidence and severity. Allegranza, in a preliminary report (1959), noted that irradiation accelerated the onset of the experimental disease and led to " a somewhat more severe symptomatology ". He pointed out that his results were " not in favour of the allergic hypothesis . . . (of aetiology of EAE) . . . according to our present knowledge about the influence of the X-rays on allergic and immunologic phenomena ". However, the present work gave no evidence that 150 r suppress the immune mechanism in the guinea-pig since skin sensitivity to the injected antigen was still developed. Uhr and Scharff (1960) working with the same animal similarly found that whole body exposure to 200 r, 24 hr. before injection of diphtheria toxoid did not suppress the development of delayed type hypersensitivity, though the intensity of the reaction was reduced.

The failure of such treatment to prevent EAE cannot therefore be urged as an argument against the " allergic " nature of the condition. Moreover, it is possible that other time relationships between irradiation and inoculation may prove more effective than those observed on the present work. Thus Cummings, Hudgins, Patnode and Bersack (1955) claimed that 130 r whole body irradiation 4 days before injection of dead tubercle bacilli with Freund's adjuvant reduced tuberculin skin sensitivity 30 days later.

The guinea-pig would appear to be an animal in which suppression of the immune responses by means of X-irradiation is difficult, the dose required being little removed, if at all, from the lethal dose (Lennox, Dempster and Boag, 1952). It is perhaps for this reason that less information is available on the effects of X-irradiation on guinea-pigs than on rabbits. However, Burrows, Deupree and

Moore (1950) reported that whilst antibody response was sharply reduced by pre-immunization irradiation, irradiation with 200 r (and to a slightly lesser degree with 100 r) 1–3 days before immunization acts as a slight stimulus to antibody formation.

It was impossible to place a reliable interpretation upon the results obtained with 300 r in the present work, since the poor general condition of animals which survived this dosage made it very difficult to assess clinical signs of EAE. However, there was no marked worsening effect and the same applied to the few animals which survived 450 r. It seems possible then that in the relatively X-ray resistant guinea-pig a dose of 150 r may be stimulatory whilst depressant doses are at the lethal or near lethal level.

SUMMARY

The effect of 150 r whole body irradiation up to the development of experimental allergic encephalomyelitis in 29 guinea-pigs has been compared with that in 32 untreated animals inoculated with the same weakly pathogenic mixture. In place of the expected protective effect a worsening was observed. There was no significant diminution of delayed sensitivity to the original human brain material used for inoculation ; these findings cannot therefore be urged against the " allergic " hypothesis of experimental encephalomyelitis.

The author would like to thank Dr. F. T. Farmer who irradiated the animals and supplied technical details, and Miss M. R. Hall for histological preparations. The work was supported by a grant from the Medical Research Council.

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FLUORESCENT ANTIBODY STUDIES IN HUMAN THYROIDITIS: AUTO-ANTIBODIES TO AN ANTIGEN OF THE THYROID COLLOID DISTINCT FROM THYROGLOBULIN

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Received for publication January 24, 1961

OF some 300 patients with Hashimoto's disease tested for circulating thyroid auto-antibodies by the precipitin reaction in agar (PP), the tanned red cell agglutination test (TRC) and the complement fixation test (CFT), about 95 per cent gave positive results; in two-thirds of cases all three tests were positive and usually of high titre (Roitt and Doniach, 1960). The first 2 tests demonstrate antibodies to thyroglobulin, the principal protein of the acinar colloid. The CFT detects a separate auto-immune system in which the antigen has been localized to the " microsome " fraction of the acinar epithelial cells.

In the small number of patients in whom all 3 serological tests proved persistently negative, the clinical and histological findings could not be distinguished from those in patients with high antibody levels. The finding of high serum γ-globulin values in these " negative " cases was highly suggestive of the presence of circulating antibodies not detectable by the tests normally used. It was therefore decided to apply the fluorescent antibody technique (Coons, 1958). Treatment of human thyroid gland sections with patients' serum followed by a fluorescent anti-human-γ-globulin conjugate (sandwich technique) revealed the presence of antibodies to a second antigen of the thyroid colloid (CA2) distinct from thyroglobulin.

The fluorescent test was applied to sera from patients with a variety of thyroid diseases, to relatives of Hashimoto cases and to mothers of athyreotic cretins. It has proved of particular interest in genetic studies of thyroid auto-immunity.

MATERIALS AND METHODS

Patients

Patients with thyroid diseases showing no detectable antibodies by TRC or CF tests.—In some Hashimoto cases histological proof was obtained by biopsy; in others the diagnosis was first made at operation and antibody tests were performed on serum specimens taken within 2 weeks of thyroidectomy. In cases of thyrotoxicosis and primary myxoedema the clinical diagnosis was supported by ^{131}I studies, BMR and serum cholesterol levels.

Thyroid patients with CF antibodies but low titre (up to 250) or negative TRC.—The Hashimoto cases were diagnosed by the thyroid function tests mentioned above with the addition of sero-flocculation reactions and electrophoretic examination of the serum proteins. Histological confirmation was not always available but the presence of high titre CFT and the shrinkage of the goitre in response to thyroid hormone therapy were used as indirect evidence of the diagnosis.

Hashimoto patients with positive precipitins (86).

Relatives of Hashimoto patients and mother and cretin-baby pairs.—Thyroid function tests were not carried out in this group.

Normal controls.—Sixty-two blood donors.

Patients' sera were stored at − 20° for 1 week to 2 yr. and were used with or without inactivation at 56°, for the fluorescent test. All sera were also tested by TRC, CFT and PP (Roitt and Doniach, 1958).

Thyroid glands

Operation specimens were used throughout, frozen within ½–2 hr. of removal. The most suitable glands were from cases of Graves' disease, though not every gland could be used for fluorescent work. In some thyroids all the follicles stained a dull green with negative sera and weakly positive results were not readily distinguishable. This was to some extent obviated by comparing the unknown with the colour of known positive and negative sera for every batch of tests. Glands with much focal thyroiditis were also unsuitable because they often contained groups of follicles which stained brightly with conjugate only. Non-toxic-nodular goitres were not deficient in antigens but in these glands the green background colour was more frequently encountered and it was more difficult to retain the colloid in the sections possibly owing to the large size of the acini. Some long-standing nodular goitres contained an abnormal substance in the colloid with a bright pale blue auto-fluorescence that did not stain with any antiserum.

Anti-human-γ-globulin conjugates

Two types of conjugate were used and found equally suitable :

1. Rabbits were immunized with purified γ-globulin obtained by sodium sulphate fractionation of human serum. The antisera were fractionated and the γ-globulin fraction conjugated with fluorescein isocyanate (Coons and Kaplan, 1950).

2. Rabbits were immunized by re-injection of their own red cells after these had been incubated with human serum for 2 hr. and washed 3 times in normal saline (Milgrom, Luszcynski and Dubiski, 1956). Conjugate was prepared by coupling fluorescein isothiocyanate (Nutritional Biochemicals Ltd.) with the γ-globulin fraction of the resulting immune serum (Marshall, Eveland and Smith, 1958). The conjugate was dialysed against buffered saline for one week at 4° to remove free fluorescein and preserved with 1/10,000 Merthiolate.

Both conjugates were absorbed with acetone extracted guinea-pig liver powder (100 mg./ml.) to remove non-specific colloid staining. A better yield of conjugate was obtained if the liver powder was first moistened with buffer, the resulting dilution having no significant effect on staining when high titre conjugates were used. Acetone extracted human thyroid powder was found to block all staining unless thoroughly washed with saline since the extracts probably contain an appreciable amount of γ-globulin.

Preparation of sections

Two methods were used and gave comparable results :

Freeze-substitution (*Balfour*, 1961).—Cubes of fresh thyroid 1–2 mm. in size were snap-frozen in liquid propane at − 180°, followed by freeze-substitution for 24 hr. in dried ethyl alcohol at − 70°. They were then embedded *in vacuo* in alcohol-soluble polyester wax (Nonex 76, Union Carbide Co.) at 40·5°. Sections (3–6 μ) were cut on an ordinary microtome and floated on absolute alcohol directly applied to the slide.

Frozen sections.—Cubes of fresh thyroid 5–10 mm. in size were snap-frozen in tubes cooled to − 70° with a solid CO_2-alcohol mixture and stored in screw-cap bottles under solid CO_2 for up to 4 weeks. Towards the end of this period there was a tendency to non-specific colloid staining with negative sera and a loss of antibody-binding power in the antigens of the colloid. Sections (6 μ) were cut in a Pearse cryostat at − 10°, taken off the knife with slides at room temperature, dried in air by a fan and fixed for 1 hr. in absolute methanol at room temperature. To prevent the colloid falling out during staining it was found necessary to dip the sections briefly (about 5–10 sec.) in 70 per cent methanol followed by buffer before applying the test serum. The length of time of this extra fixation is rather critical. If the section is left in 70 per cent alcohol for too long, the antigens are destroyed and poor staining is obtained ; on the other hand if the sections are dipped too quickly the colloid tends to fall out. This tendency varied a great deal from one gland to another.

Although the dip in 70 per cent methanol leads to weaker staining it has so far proved the best method of keeping the colloid in the sections.

The freeze-substitution method has the double advantage of not requiring a cryostat and of producing histological blocks which can be kept without deterioration of the colloid antigens for at least 3 months, an important consideration if thyroidectomy specimens are not readily available. However cryostat sections are easier to prepare and they have the added advantage of being suitable for the detection of antinuclear factors (ANF test) and of alcohol sensitive cytoplasmic antigens (Holborow, Brown, Roitt and Doniach, 1959).

Staining procedure

Barbitone buffered saline, pH 7·2 was used (0·02 M—barbitone buffer: Na barbitone 20·6 g., 0·1 N/HCl, 80·6 ml., NaCl 85·0 g., distilled water to 5 l., kept at 4° and diluted with an equal volume of water before use). Test sera were applied to the sections for 20–30 min. in a moist chamber and washed off in running buffer. The slides were left in buffer for 20 min., wiped close to the section and a small drop of conjugate run into the residual buffer (drying was avoided at this stage). Conjugate was left on for 20 min. followed by a second wash of 20 min. The sections were mounted in 50 per cent glycerol buffer. This was found more suitable than the 90 per cent glycerol normally used as it avoids the diffusion of fluorescein from the colloid and allows stained sections to be kept for 3–4 days at 4°.

Fluorescent microscopy

Two different systems were in use in the two Institutes in which the work was carried out.

1. A carbon-arc light source was used at Mill Hill. The light was focussed on to the back of the dark-field condenser in a horizontally placed microscope and screened with a solution of ammoniacal copper sulphate. An Ilford gelatin filter No. 108 was used in the ocular. A drop of glycerol was placed between the condenser and the slide for viewing.

2. A Zeiss microscope fitted with a high pressure mercury vapour lamp (Osram HBO 200) was used at the Middlesex Hospital and found to give almost as good illumination as the carbon arc. The light source was screened with a blue glass filter (UG 5, Schott and Co., Mainz) of maximum transmission at 300 mμ. The beam was deviated into the microscope by a surface aluminized mirror and a dark ground condenser with glycerol immersion was used as for the carbon-arc. A double thickness of a colourless gelatin filter, Wratten 1A or 2B was used as a secondary filter in the ocular.

RESULTS

Fluorescent staining of thyroid sections

With the filters used the normal colloid, in sections stained with conjugate only, gives a blue colour and with negative serum a blue-green or bottle green colour according to the glands used. Plasma in blood vessels shows bright green staining due to γ-globulin. There is some non-specific staining of the basement membranes and connective tissue fibres but this does not interfere with the interpretation of acinar staining.

In many blocks of thyrotoxic thyroid glands, isolated follicles or groups of acini gave a bright uniform staining of the colloid in control sections treated with conjugate only (Fig. 1). These reacting acini were sometimes large, showed breaches of continuity in the lining epithelium and contained phagocytic cells. They were often situated in areas of lymphocytic infiltration. In other cases there were also groups of small acini staining with conjugate which did not look obviously injured and were not in close contact with lymphoid germinal centres. Repeated absorption of the conjugates failed to affect the focal staining and it was concluded that this was due to the presence of γ-globulin, most probably antibodies to the colloid, bound *in vivo*. When assessing the specificity of conjugates or the staining power of unknown sera, occasional staining follicles should be disregarded. The

appearance of normal acini must be judged in areas free of focal thyroiditis and a serum can be considered positive only if it stains every follicle in the section.

Staining of colloid by Hashimoto sera in the sandwich technique was of three different patterns one of which was obtained with sera having no other detectable antibodies. The patterns could be obtained with adjacent sections from the same thyroid gland and were only dependent on the patient's serum provided the antigens were not injured in fixation. The first was a 3-dimensional appearance with floccules having bright edges and dark centres. (Fig. 2.) On focussing up and down this appeared to be due to the formation of large aggregates, presumably of immune complexes. This pattern was only obtained with sera giving positive precipitins in agar. The floccules were brightest and largest when strong precipitating sera were applied to unfixed sections and the colloid was retained by the immune aggregates. On the whole the floccules were less marked in the freeze-substituted sections than in frozen sections fixed for 1 hr. in methanol. Secondly a " crazy-paving " pattern in which the background was dull green, the acinar edge was brightly stained and there were bright cracks throughout the colloid, (Fig. 3) was seen with precipitating sera when the thyroglobulin was partly denatured by treatment with 70 per cent alcohol, or, in the absence of denaturation, with sera having negative agar tests but relatively high TRC titres (over 2,500). Lastly a flat bright green uniform staining of the entire colloid (Fig. 4) was obtained with Hashimoto sera giving negative results by other tests for thyroid antibodies,and was strongly suggestive of an antibody directed against a second colloid antigen (CA2) distinct from thyroglobulin. Occasionally cracks were seen in the colloid with sera which failed to agglutinate tanned red cells. In some of these instances the staining was uniform without a bright edge and it is thought that the cracks were an artefact, since splitting of the colloid was sometimes seen with known anti-CA2 sera when using thyroid blocks which were beginning to deteriorate. In other sera with a negative TRC test, a true crazy-paving pattern was obtained and there is suggestive evidence (to be presented elsewhere) that such sera may react with yet another unidentified antigen of the thyroid colloid.

Characterization of CA2 Antibodies

The following experiments were carried out to provide confirmatory evidence for the existence of a second colloid antibody. For these experiments we chose the sera of patients with Hashimoto's disease proved by biopsy who had raised γ-globulins and high sero-flocculation values and who invariably gave bright

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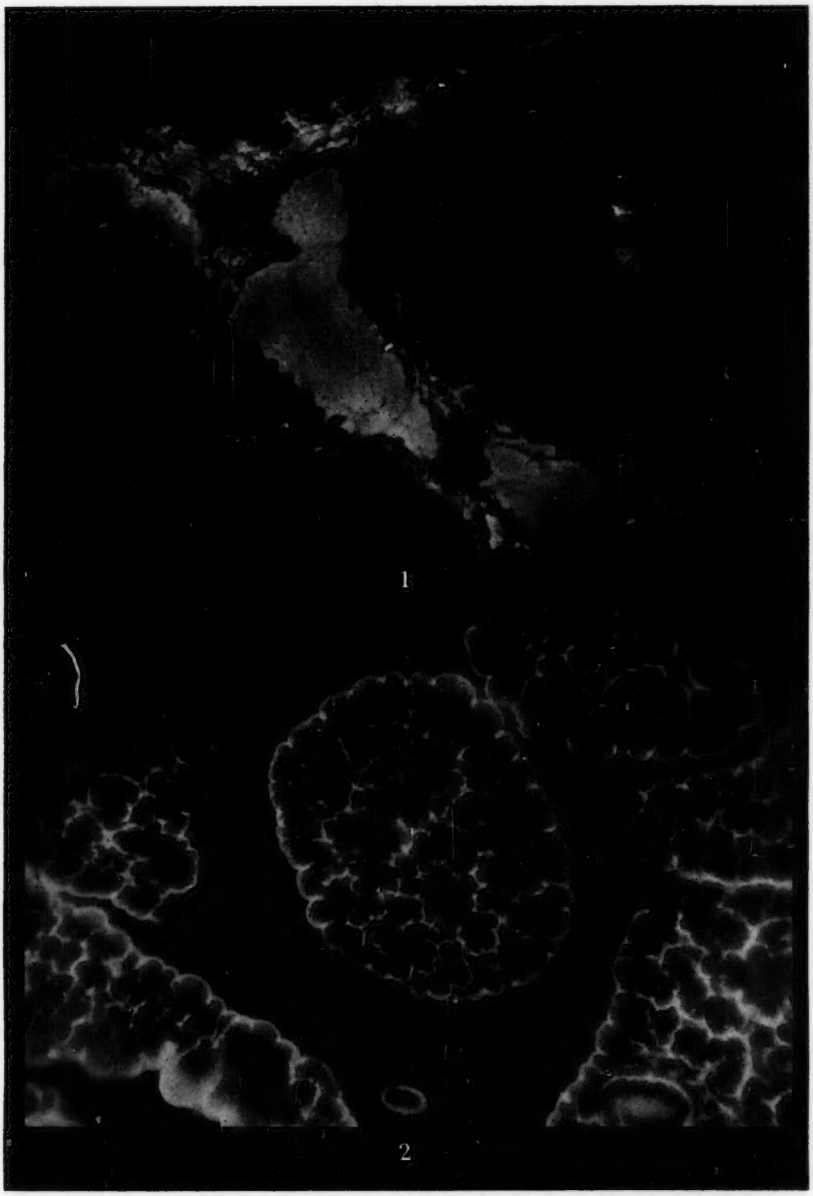
EXPLANATION OF PLATES.

FIG. 1.—Thyroid gland from a patient with focal thyroiditis stained directly with fluorescent rabbit anti-human-globulin conjugate. Section shows fluorescence in isolated acini containing macrophages. This is probably due to auto-antibodies bound to the colloid *in vivo*.

FIG. 2.—Human thyroid gland stained by the fluorescent sandwich technique. A precipitin positive Hashimoto serum was applied, followed by fluorescein conjugated rabbit anti-human-globulin serum. The precipitin aggregates produce a 3-dimensional floccular appearance in the colloid space. There is no staining of the thyroid epithelial cells or nuclei.

FIG. 3.—Test as in Fig. 2 but with precipitin negative Hashimoto serum having a TRC titre of 25,000. No floccules are formed. The colloid space is stained dull green with a brighter edge and bright cracks.

FIG. 4.—Test as in Fig. 2. The Hashimoto serum used was negative in the preciptin, TRC and CF tests. There is uniform bright apple-green staining of the colloid space.



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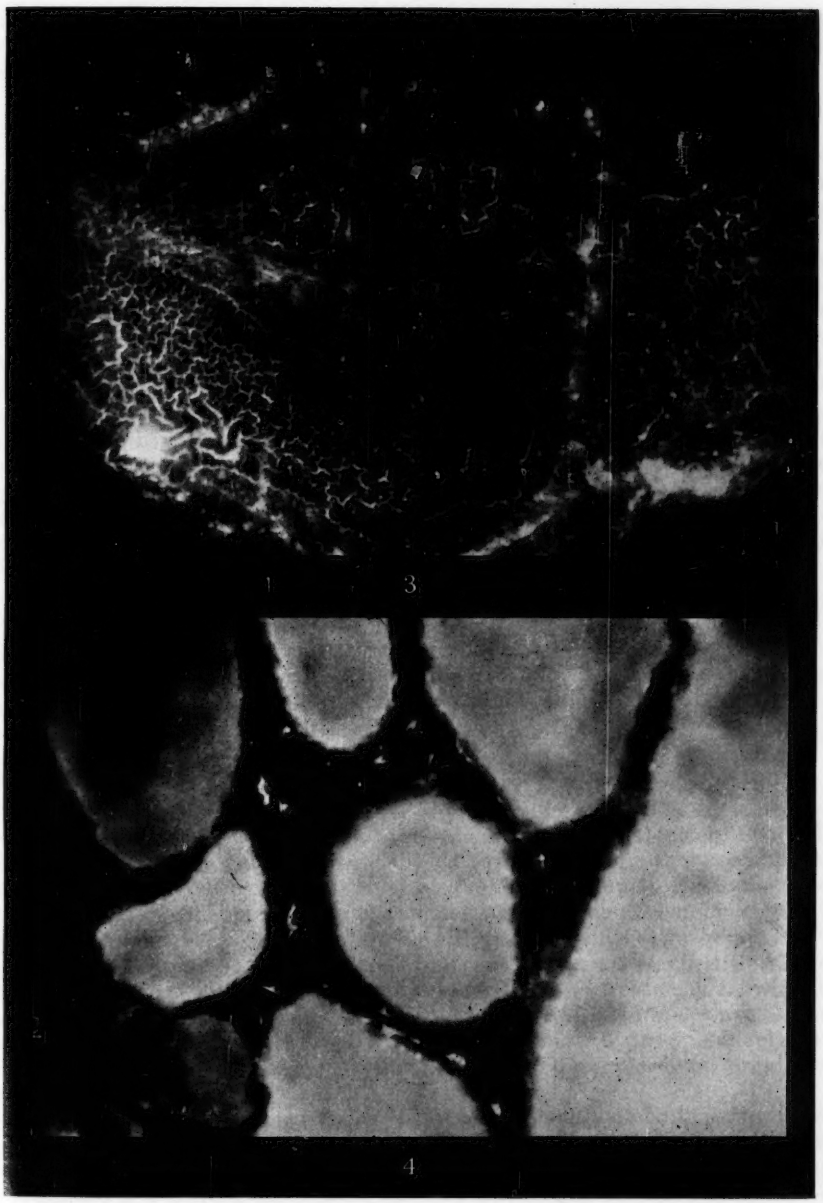

Balfour, Doniach, Roitt, Couchman.

fluorescent staining of the uniform pattern though negative results were always obtained by TRC and CFT. Precipitin tests in agar were negative both against purified thyroglobulin and crude extracts of thyrotoxic and non-toxic glands.

Thyroglobulin absorption

Sera with CA2 antibodies could be absorbed with up to 50 mg. of thyroglobulin per ml. without loss of specific fluorescent staining whereas many precipitin positive sera became negative in the Coons' test after absorption with sufficient thyroglobulin to reverse their tanned cell agglutination.

Since it was likely that some precipitin positive sera also contained CA2 antibodies a series of 50 precipitating Hashimoto sera all giving the floccular pattern were absorbed with 50 mg. of thyroglobulin per ml. and re-tested by the Coons' technique. Nineteen (38 per cent) of these sera now gave the uniform pattern characteristic of CA2 antibody though the TRC test had become negative as a result of absorption. It is likely that the incidence of CA2 antibodies in precipitin positive sera is even higher than these figures would indicate since we have found that purified thyroglobulin prepared by the salting out method of Derrien, Michel and Roche (1948) is contaminated with CA2 antigen. In fact, weak anti-CA2 sera can lose their colloid staining power after absorption with 50 mg./ml. of thyroglobulin owing to this contamination. On the other hand, the fluorescent staining of strong anti-CA2 sera can easily be reversed when they are absorbed with crude thyroid extract instead of thyroglobulin.

Test for blocking antibody

Thyroglobulin coated tanned cells were incubated with a 1/10 dilution of anti-CA2 serum and were then put up with several TRC positive sera. No decrease in agglutination titre was obtained showing that no blocking antibody to thyroglobulin was present.

Radioactive co-precipitation

Anti-CA2 sera were also tested jor thyroglobulin antibodies by a sensitive co-precipitation method using [^{131}I] thyroglobulin (Roitt and Doniach, 1959). The sera were allowed to react with the labelled thyroglobulin and any soluble complexes formed were then co-precipitated with rabbit anti-human-γ-globulin serum. After washing the final precipitate the amount of thyroglobulin combined with the patients' serum could be estimated by the radio-activity of the precipitate. In the case of anti-CA2 sera the activity of the co-precipitate was not significantly different from that obtained with normal sera subjected to the same treatment, showing that no thyroglobulin had been bound by anti-CA2 sera. The experiment was repeated using whole thyroid extract from a patient receiving ^{131}I pre-operatively, with similar negative results suggesting that no other iodine-containing protein was inplicated.

To make sure that the particular rabbit anti-human-γ-globulin serum used in the radioactive experiment reacted fully with the colloid antibodies in the patient's serum its ability to inhibit the fluorescent test was examined. The rabbit serum was applied to a thyroid section after the anti-CA2 serum and was found to inhibit completely subsequent fluorescent staining by the conjugated rabbit anti-human-γ-globulin.

Effect of fixatives

Since it is known that the antigenic properties of thyroglobulin withstand formalin fixation (Beutner, Witebsky, Rose and Gerbasi, 1958) and heating (Witebsky and Rose, 1956), precipitating and anti-CA2 sera were tested by the Coons technique using ordinary formalin fixed, paraffin embedded thyroid sections. Weak fluorescent staining of the colloid was still present with precipitin positive sera but was completely lost in the case of anti-CA2 serum showing that the second antigen is destroyed by formalin. As a control it was shown that heating a methyl alcohol fixed frozen section to 56° for 2 hr. did not affect either antigen. Picric acid in methanol produced a rather green background colour in the colloid with control negative serum but did not destroy either CA2 antigen or thyroglobulin, and the two distinct patterns of fluorescence were maintained.

Dilution of CA2 antibodies

To assess the amount of CA2 antibody present in different sera, a series of dilutions of two anti-CA2 sera were prepared varying from 1/2 to 1/128. Serial sections from a thyroid block were treated with these diluted sera and stained by the sandwich technique. It was found that the brightness of the staining diminished with increasing dilution but that the uniform pattern of staining did not change. The dilution at which staining could no longer be seen was taken to be the end point. One of the sera gave a titre of 1/32 and the other 1/64.

In a similar experiment done with a precipitating serum, the floccular pattern was intensified at 1/8 and 1/16 and thereafter the floccules gradually disappeared, giving way to the crazy-paving appearance ; the staining disappeared at 1/128. Another precipitating serum gave the crazy-paving pattern up to dilutions of 1/128 and became negative at 1/256.

Haemagglutination experiments

The TRC test is normally performed with sheep cells coated with thyroglobulin and subsequently formolinized for stabilisation (Fulthorpe, Roitt, Doniach and Couchman, 1961). Thus even though CA2 antigen is present as an impurity in thyroglobulin preparations it would not react in this system owing to its denaturation by formalin. However, anti-CA2 sera also gave negative results when tested against unformalized thyroglobulin-coated cells and against fresh tanned cells coated with whole thyroid extract.

Incidence of CA2 Antibodies

Hashimoto's disease

The incidence of antibodies to the second colloid antigen in the sera of Hashimoto patients is shown in Table I. Sixteen histologically proven cases, showing no other antibodies (Group A), all gave fluorescent colloid staining of the uniform CA2 pattern. Of 60 Hashimoto sera with CFT titres ranging from 1/32 to over 1/512 but with negative or low TRC results (up to 250) (Group B), 58 showed colloid staining of CA2 pattern. It was unlikely that any trace of anti-thyroglobulin present was responsible since sera from colloid goitre cases with comparable TRC titres failed to stain in the fluorescent test. Eighty-six precipitin positive cases all gave the floccular pattern and of the 50 sera absorbed with excess thyroglobulin (Group C), 19 changed to the CA2 pattern while the others became negative.

TABLE I.—*Incidence of CA2 Antibodies in Hashimoto Patients*

Group	Serology	Number tested	CA2 fluorescent test positive
A	CFT − ve, TRC − ve	16	16
B	CFT + ve, TRC − ve	60	58
C	Precipitin + ve Absorbed with excess thyroglobulin	50	19

Other thyroid diseases

The incidence of CA2 antibodies in other thyroid diseases and in the controls is shown in Table II. It can be seen that patients with other evidence of auto-

TABLE II.—*Incidence of CA2 Antibodies in Other Diseases and in Controls*

Diagnosis	Number tested	Proportion of cases with CA2 fluorescent test positive: Patients with TRC and/or CFT positive	Patients with TRC and CFT negative	Total positives (per cent)
Thyrotoxicosis	122	34/59	16/63	41
Non-toxic nodular goitre	68	5/7	6/61	16
Primary myxoedema (adults)	34	7/9	6/25	38
Congenital athyreotic cretinism				
Mothers	11	0/2	4/9	36
Babies	11	—	1/11	9
De Quervain's thyroiditis	3	—	1/3	33
Relatives of Hashimoto patients	53	10/25	6/28	30
Blood donors (controls)	62	0/2	2/60	3

Only sera giving TRC titres up to 250 were tested for anti-CA2 fluorescence since higher titres of thyroglobulin antibodies interfere with the interpretation of results.

immunity as shown by positive TRC or CFT results, are much more likely to give positive CA2 staining in the fluorescent test and this applies equally to all the thyroid conditions examined so far. Thus over 50 per cent of thyrotoxic patients who had a positive CFT gave fluorescent staining whereas only 20 per cent of those negative by other antibody tests had CA2 antibodies.

Of 7 nodular goitre patients with other antibodies, 5 were positive in the fluorescent test and histological examination of the gland showed lymphoid foci in every case. Of the 61 cases negative by TRC and CFT only 6 had CA2 antibodies. Thyroidectomy has not yet been carried out in all these cases but the correlation of CA2 fluorescence with lymphoid foci has been good in the 22 cases examined histologically.

Primary myxoedema patients differ from those with Hashimoto goitres in that they rarely show CA2 fluorescence if the TRC and CFT are negative so that the Coons method is not likely to provide additional help in diagnosing doubtful hypothyroidism when the thyroid is atrophic.

CA2 antibodies were found in one case of histologically proved de Quervain's disease. The test was first carried out 7 weeks after the acute onset of the disease

and was still positive 3 months after recovery. Nothing can be said of the incidence in these patients until more cases have been tested.

Genetic Studies and Controls

Fifty-three close relatives of Hashimoto cases have so far been tested by the fluorescent method and 16 (30 per cent) have given positive results. As in patients with thyroid diseases, the subjects with TRC or CFT antibodies appear more likely to have anti-CA2 ; however, in about 10 per cent of relatives only the fluorescent test was positive. (Details of this genetic study will be reported elsewhere.)

Following the suggestions of Blizzard, Hamwi, Skillman and Wheller (1959), Blizzard, Chandler, Landing, Pettit and West (1960) and of Beierwaltes, Dodson and Wheeler (1959) that athyreotic cretinism may be due to intra-uterine destruction of the foetal thyroid by cytotoxic antibodies, we tested 11 mother and baby-cretin pairs; 6 of the mothers had antibodies to at least one thyroid constituent and anti-CA2 was present in 4. Only one baby serum gave fluorescent staining and this could have been of maternal origin.

Of 62 blood donor controls, two showed a weak CA2 colloid fluorescence. none had a positive CFT and 2 others had TRC titres of 25 and 250 respectively.

DISCUSSION

The presence of antibodies to the thyroid colloid detected by Coons' method in patients whose sera fail to react in the TRC. CFT and precipitin tests was noted by other authors (Blizzard *et al.*, 1960) beside ourselves (Doniach, Hudson and Roitt, 1960). The published reports on fluorescent staining of colloid (White, 1957 and 1959; Hiramoto, Engel and Pressman, 1958; Landing, West and Esselborn, 1958) do not comment on the occurrence of varying patterns of staining or on the possibility of there being 2 distinct antibodies to the thyroid colloid. In the case of Beutner *et al.* (1958), formalin fixed sections were used and this would have destroyed CA2 antigen.

The uniform pattern of staining obtained with sera showing no other positive antibody tests suggested that an antigen distinct from thyroglobulin and " microsomal " antigen was involved. The absence of floccules further suggested a non-precipitating antibody. The fact that these sera did not give precipitins either with thyroglobulin or with crude extracts and their failure to agglutinate freshly prepared tanned red cells in spite of the presence of CA2 in the thyroid extracts suggested that these sera either contained a non-agglutinating type of antibody or that CA2 failed to coat the cells. The possibility of non-agglutinating antibodies against thyroglobulin, was excluded by the radioactive co-precipitation and blocking experiments. The character of the CA2 antibodies should be further investigated by coating tanned red cells with a purified CA2 fraction. The distinct nature of CA2 and thyroglobulin was confirmed by applying a conjugated Hashimoto anti-CA2 serum to eluates of thyroid extracts separated on DEAE-cellulose columns, when a fraction containing CA2 was shown to be free of thyroglobulin as judged by immunological criteria (Wilson, Roitt and Doniach, unpublished). The possibility that CA2 may be one of the thyroid proteases is at present under investigation.

The floccular three dimensional fluorescent pattern seen only with precipitin positive sera is likely to be produced by the same mechanism which forms visible

aggregates in the test tube. Conjugated precipitating sera give the floccular pattern when the direct Coons method is used. The floccules obtained in the sandwich technique are also produced by the patient's serum and not by the superimposed rabbit conjugate. This was shown by staining with amido-black a frozen thyroid section treated with Hashimoto serum. Dark blue floccules were seen in the colloid when precipitin positive serum was used but not with anti-CA2 sera. When precipitin positive serum is applied to sections with marked focal thyroiditis it produces the floccular pattern in all the acini including those which on adjacent sections stained uniformly with conjugate only. This implies that antibody bound *in vivo* does not block all the combining sites in the colloid.

The CA2 colloid auto-antibody described in this study appears to be as common in Hashimoto patients as the thyroglobulin and microsomal antibodies. Thyroglobulin antibodies are detectable in over 85 per cent of Hashimoto patients and a similar percentage of cases give positive CFT reactions though the two antibodies do not necessarily occur together. About 5–8 per cent of cases are negative by TRC and CFT tests and all of these have so far been positive by the fluorescent test. Thus using all present available methods, autoantibodies have so far been demonstrated in 100 per cent of untreated Hashimoto cases subsequently proved by histology.

It is more difficult to determine the incidence of CA2 antibodies in sera with precipitins but the absorption experiments suggest that the 2 antibodies frequently co-exist.

The CA2 antibody is also present in thyrotoxic and other thyroid patients who have lymphocytic foci in their gland. Its presence in the asymptomatic relatives of Hashimoto patients with apparently normal thyroids is of theoretical interest since it adds to the evidence obtained by Hall, Owen and Smart (1960) on the genetic predisposition to the development of thyroid auto-immunity. It is not yet known whether these relatives have focal lymphoid infiltration in the thyroid. The CA2 antibody is rarely found in advanced primary myxoedema when presumably little if any colloid antigens remain in the gland.

The antibody appears to be distinct from the cytotoxic factor of Hashimoto sera (Pulvertaft, Doniach, Roitt and Hudson, 1959) since several sera negative by the fluorescent test were cytotoxic to thyroid tissue cultures. It is probable that the CA2 antibody is not directly implicated in causing thyroiditis lesions, but is formed as a result of the follicular disruption as are the other circulating antibodies of Hashimoto's disease.

Clinically the fluorescent test is of diagnostic interest in Hashimoto patients who fail to show antibodies by any other tests. Although in some instances the γ-globulin and sero-flocculation reactions are abnormally raised, this is by no means the rule, so that the Coons method provides useful specific evidence of the presence of auto-immunization. Unlike the precipitin reaction the fluorescent test is not diagnostic of Hashimoto's disease and can only be used in support of suggestive clinical features and suitable ^{131}I studies. However, if the fluorescent test as well as the TRC and CFT are all negative, the diagnosis of Hashimoto's disease is practically excluded.

SUMMARY

The serum from the 5–8 per cent of Hashimoto patients showing no antibodies to thryoglobulin or the microsomal antigen " stain " the colloid of human thyroid

sections using Coons' sandwich technique and produce a different pattern from that of anti-thyroglobulin. The antigen CA2 was distinguished from thyroglobulin by its sensitivity to formalin fixation, by a radioactive co-precipitation technique, and by absorption studies with thyroglobulin. Anti-CA2 antibodies are present in most Hashimoto cases but are also found in other thyroid diseases and are not diagnostic of lymphadenoid goitre.

We are grateful to Dr. J. H. Humphrey, Professor Sir Charles Dodds, Professor F. Dickens and Mr. R. Vaughan Hudson for their encouragement and interest in this work. We thank Professor A. Kekwick and the Clinical Research Committee for the facilities provided at the Institute of Clinical Research. We are greatly indebted to the surgeons who kindly gave us thyroidectomy specimens: Mr. J. E. Piercy, Mr. C. J B. Murray and Mr. R. Turner-Warwick. Our thanks are due to Miss Lorna Franklin for technical assistance.

The work is supported in part by the British Empire Cancer Campaign. The Zeiss microscope and the cryostat were generously given to the Middlesex Hospital research group by the Mary Kinross Charity Fund.

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THE OXYGEN REQUIREMENT FOR POLIOVIRUS REPLICATION IN CONCENTRATED SUSPENSIONS OF ERK CELLS

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Received for publication January 26, 1961

A SYSTEM for the production of high titre poliovirus in concentrated suspensions of Porton embryo rabbit kidney (ERK) cells was described by Westwood, Appleyard, Taylor-Robinson and Zwartouw (1960). It was shown further (Zwartouw, Taylor-Robinson and Westwood, 1960) that virus yields were reduced little, if at all, by the omission from the suspending medium of all nutrients including glucose. Under these conditions acid production in the concentrated cell suspensions was minimal and the pH of cultures was easily controlled. These findings were valid for experiments carried out with 10 ml. volumes of suspension containing 10^7 cells/ml. in bottles of 250 ml. capacity agitated by rolling. However, if either the volume of suspension or the cell concentration was increased the virus yield fell abruptly. This fall in virus yield was traced to a deficiency of cell oxygenation. The conditions of oxygenation required by cells suspended in the glucose-free medium and infected in suspension were, therefore, studied. These studies extended to a determination of the requirements for poliovirus production in stirred cultures up to 1·5 l. in volume and at cell concentrations of up to 10^8 cells/ml.

MATERIALS AND METHODS

Cells

The Porton ERK 1 cell line, derived from embryo rabbit kidney (Westwood, Macpherson and Titmuss, 1957) was grown in Roux bottle monolayer cultures and the cells harvested as described previously (Westwood *et al.*, 1960).

Virus

Stocks of Polivirus Type I (Brunhilde), Type II (Lansing) and Type III (Leon) were prepared by the technique described previously (Zwartouw *et al.*, 1960) and stored at − 60°. Type I was used for all experiments except where stated otherwise.

Assay methods

Poliovirus infectivity titrations were carried out with ERK cells by Cooper's (1955) modification of Dulbecco's plaque technique and the results expressed as plaque forming units (PFU)/ml.

pH was measure with a glass electrode.

Poliovirus growth procedure

The harvested ERK cells, centrifuged from trypsin +EDTA (ethylenediamine-tetra-acetic acid) suspension, were washed with ES-G (Earle's saline, without glucose, buffered with

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0·11 per cent sodium bicarbonate and gassed with 5 per cent v/v CO_2 in air) and then resuspended in ES-G. Cells in suspension were infected with poliovirus by adding 2 PFU/cell and any further desired additions (*e.g.* glucose) were made before the mixture was finally diluted to the required cell concentration. All cultures were incubated for 24 hr. at 37° and agitated on a roller drum (20 r.p.h.), a rotary shaker (165 r.p.m., 2·2 cm. radius) or stirred with magnets. After incubation, cultures were cooled to 4°, centrifuged at 2000 *g* for 10 min. and the supernatant fluid assayed for virus infectivity titre.

EXPERIMENTAL AND RESULTS

The alternative requirement for oxygen or glucose

Early experiments indicated that oxygenation was a limiting factor for poliovirus production by ERK cells suspended in saline without glucose. However, further investigation proved that oxygen was essential for virus replication only when glucose was absent but if sufficient glucose was present virus replicated in anaerobic cultures (Table I). A sufficient supply of glucose for anaerobic suspen-

TABLE I.—*Alternative Oxygen or Glucose Requirement for Poliovirus Replication**

Gas phase 5 per cent v/v CO_2 + 95 per cent	Glucose in medium (per cent)	Virus titre (PFU/ml.)	
		Expt. 1	Expt. 2
Air	0	$2·2\times10^9$	$1·7\times10^9$
N_2	0	$<5\times10^6$	$<5\times10^6$
N_2	0·4†	$2·4\times10^9$	$1·5\times10^9$

* 10 ml. volumes of suspension (10^7 cells/ml. in ES-G) were agitated in 250 ml. Pyrex bottles on the rotary shaker.

† Sodium bicarbonate concentration was increased to 0·44 per cent.

sions containing 10^7 cells/ml. was 0·4 per cent ; lower concentrations reduced the virus yield and higher concentrations did not alter it.

Cultures without glucose and requiring oxygen

The omission of glucose from the cell-suspending fluid in concentrated cell suspensions greatly facilitated the control of pH without adversely affecting the virus yield from small volumes (10 ml.) of culture (Zwartouw *et al.*, 1960). This advantage prompted an investigation of the problem of providing adequate oxygenation in larger cultures without glucose and at higher cell concentrations.

The effect of oxygen concentration.—Table II shows the effect on virus yield of various oxygen concentrations in the gas phase of cultures. With 10 ml. of 10^7 cells/ml. in a 250 ml. bottle maximum virus yields were obtained over a range of oxygen concentrations from 5–95 per cent v/v. Some virus replication occurred at 1 per cent oxygen but no virus was produced with 0·5 per cent or less. At a lower cell concentration (10^6 cells/ml.) maximum virus yields were obtained with 5–50 per cent v/v oxygen and virus production was completely suppressed at 0·1 per cent. There was some indication that too great an excess of oxygen was inhibitory ; the virus yield was not maximal from 10^6 cells/ml. in contact with a gas phase of 95 per cent oxygen. It is relevant to note that Zwartouw and Westwood (1958) showed that oxygen concentrations above 15 per cent v/v in the gas phase of agitated monolayer tissue cultures exerted a " toxic " effect and decreased cell multiplication.

TABLE II.—*Effect of Oxygen Concentration in the Gas Phase on Poliovirus Production by Cells Suspended in Glucose-free Saline**

Oxygen in gas phase† (per cent v/v)	Virus titre (PFU/ml.) With 10^7 cells/ml.	Virus titre (PFU/ml.) With 10^6 cells/ml.
0	$<5\times10^6$	$<5\times10^5$
0·1	$<5\times10^6$	6×10^5
0·5	$<5\times10^6$	$2\cdot3\times10^7$
1·0	$2\cdot3\times10^8$	$1\cdot8\times10^7$
5·0	$9\cdot9\times10^8$	$6\cdot8\times10^7$
20	$9\cdot2\times10^8$	$5\cdot6\times10^7$
50	$1\cdot2\times10^9$	$5\cdot0\times10^7$
95	$1\cdot1\times10^9$	$2\cdot6\times10^7$

* 10 ml. volumes of cell suspension in ES-G were agitated in 250 ml. Pyrex bottles on the rotary shaker.

† All gas phases contained 5 per cent v/v CO_2 and nitrogen to 100 per cent.

Increased culture volume.—The primary object of this part of the investigation was to determine whether poliovirus could be produced in concentrated cell suspensions on a larger scale. For practical reasons, agitation of cultures on the roller drum or shaker was replaced by stirring in a stationary vessel. Conditions were arranged to provide the maximum oxygen solution rate attainable by simple means and the largest culture volume which produced high virus yields in a particular culture vessel was determined. Cultures were placed in 250 ml. bottles over magnetic stirrers and agitated with magnets (length 2·5 cm.) at 450–600 r.p.m. with a gas phase of oxygen containing 5 per cent v/v CO_2. (Faster stirring speeds produced low yields of virus probably due to mechanical damage of the cells.) Under these conditions, high titre virus was regularly produced from 50 ml. and occasionally from 100 ml. of cell suspension. These volumes represented fluid depths of 2·5 and 5·0 cm. respectively in the vessel used. Similar conditions were then used on a larger scale by increasing the volume of cell suspension and the size of the culture vessel simultaneously so that the fluid depth did not exceed 5·0 cm. The examples in Table III show that all three types of poliovirus were obtained

TABLE III.—*Production of High Titre Poliovirus in Stirred Cell Suspensions with Oxygen and Glucose-free Saline**

Virus type	Expt. No.	Cell suspension Volume (ml.)	Cell suspension Fluid depth (cm.)	Culture vessel† capacity (ml.)	Virus titre (PFU/ml.)
I	1	230	3·6	1,000	$2\cdot5\times10^9$
	2	450	4·0	2,000	$1\cdot6\times10^9$
	3	1,500	4·8	10,000	$1\cdot6\times10^9$
II	4	200	3·3	1,000	4×10^8
	5	530	4·6	2,000	5×10^8
III	6	274	2·4	2,000	$1\cdot3\times10^9$
	7	400	3·6	2,000	$1\cdot3\times10^9$

* 10^7 cells/ml. were suspended in ES-G and agitated with magnetic stirrers (magnet length 5 cm.) at 450–600 r.p.m. The gas phase in the culture vessels was oxygen containing 5 per cent v/v CO_2.

† Height of vessels was 1·5 to 2 × diameter.

at high titre and that the culture volume could be increased to 1·5 l. The yields of type II virus were lower than types I and III ; this agreed with the relative yields reported by Sheffield and Churcher (1957) for the same three virus strains in ERK cells.

Increased cell concentration.—Previous attempts to obtain high titre virus from cell concentrations above 10^7/ml. in suspensions containing glucose were considered to have failed because of the pH fall due to the rapid production of acid (Westwood

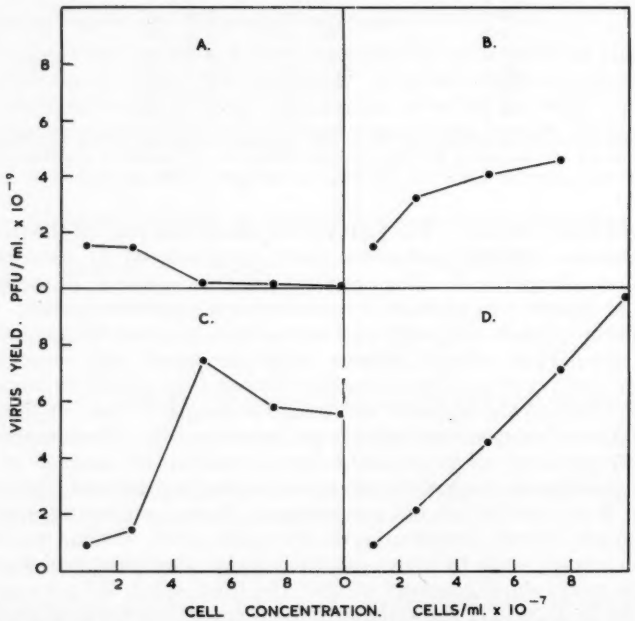

FIG.—Effect of increased oxygenation on virus yields from high cell concentrations. Five ml. of cell suspension in ES-G was agitated in a 250 ml. bottle with a gas phase containing 5 per cent v/v CO_2 and 95 per cent as follows : A, air with gentle agitation (on roller drum) ; B, air with vigorous agitation (on rotary shaker) ; C. oxygen with gentle agitation ; D, oxygen with vigorous agitation.

et al., 1960). This difficulty was overcome by omitting glucose from the cell-suspending fluid and providing adequate oxygenation. The figure shows that as oxygenation was increased in stages higher cell concentrations were able to produce maximal yields of virus. With 95 per cent v/v oxygen in the gas phase and vigorous agitation on the rotary shaker cell concentrations of 10^8/ml. produced 10^{10} PFU/ml. of virus.

Control of pH at high cell concentrations.—Zwartouw *et al.* (1960) showed that suspensions of 10^7 cells/ml. in the absence of glucose produced maximal yields of virus between pH 6·8 and 7·7 provided that the sodium bicarbonate concentration was in the range 0·07–0·22 per cent. The bicarbonate concentration (0·11 per cent) adopted for suspensions of 10^7 cells/ml. was insufficient to prevent cultures of

higher cell concentration from falling below pH 6·8 and it was necessary to redetermine the optimum amount of bicarbonate. Table IV shows that the increase

TABLE IV.—*Effect of Bicarbonate Buffer Concentration on Virus Yields from* 5×10^7 *and* 10^8 *Cells/ml. Suspended in ES-G**

Cell conc. (cells/ml.)	$NaHCO_3$ conc. (per cent)	CO_2 in oxygen (per cent v/v)	Final pH of cultures†	Virus titre (PFU/ml.)
5×10^7	0·11	0	6·5	$<5 \times 10^6$
	0·22	5	6·9	5×10^9
	0·33	5	7·0	$4{\cdot}5 \times 10^9$
	0·44	10	7·0	4×10^9
	0·66	15	7·1	3×10^7
10^8	0·22	5	6·8	$8{\cdot}4 \times 10^9$
	0·33	5	6·8	1×10^{10}
	0·44	10	6·8	1×10^{10}
	0·66	15	7·0	2×10^9

* See note to Table II.
† Initial pH of cultures was $7{\cdot}3 \pm 0{\cdot}1$.

required for maximal virus yields was not in proportion to the 5 and 10 fold increase made in cell concentration and that 0·22 to 0·44 per cent bicarbonate was the optimum range.

The oxygen requirement in large culture volumes with glucose

Sufficient buffer can be added to control the pH adequately when 10^7 cells/ml. are metabolising glucose. Since small volumes (10 ml.) were able to replicate poliovirus anaerobically, larger volumes of cell suspension with glucose were tested to see if this overcame the problem of oxygenation and enabled deeper cultures to be used. However, experiments showed that maximal virus production was not obtained in deeper cultures containing 10^7 cells/ml. and 0·4 per cent glucose unless oxygen was also supplied. The oxygen requirement was less than in the absence of glucose so that in a 250 ml. bottle 100 ml. of cell suspension with air in the gas phase and 150 ml. of cell suspension with oxygen in the gas phase both produced high titre virus (Table V). This represented a 2–3 fold increase of volume (or depth of culture) over the 50 ml. which could be used in the same vessel in the absence of glucose.

TABLE V.—*Oxygen Requirement of Larger Culture Volumes Containing* 10^7 *Cells/ml. and Glucose**

Experiment No.	Volume of culture (ml.)	Gas phase†	Virus titre (PFU/ml.)
1	50	Air	$2{\cdot}1 \times 10^9$
	100		$1{\cdot}6 \times 10^9$
	150		3×10^8
2	150	N_2	$1{\cdot}2 \times 10^8$
		Air	2×10^8
		O_2	$1{\cdot}1 \times 10^9$

* Suspensions containing 10^7 cells/ml., 0·4 per cent glucose and 0·44 per cent sodium bicarbonate were stirred by magnets at 450–600 r.p.m. in 250 ml. bottles.
† All gas phases contained 5 per cent v/v CO_2.

DISCUSSION

In small volumes of cell suspension poliovirus replicated if either adequate glucose or adequate oxygen was supplied. Presumably the cells contained sufficient endogenous substrate to provide the energy for virus synthesis by oxidative processes but not by anaerobic metabolism. The latter process could provide the necessary energy when sufficient glucose was present in the cell-suspending fluid. This is in agreement with the observation of Gifford and Syverton (1957) who found that poliovirus replication occured anaerobically in HeLa cells with a tissue culture maintenance medium presumably containing glucose. On the other hand, a discrepancy was found with larger culture volumes containing glucose which did not produce maximal yields of virus unless oxygen was also supplied. One possible explanation is that the smaller ratio of gas phase volume to liquid volume caused a " toxic " concentration of bicarbonate ions to be retained in solution. Zwartouw *et al.* (1960) showed that buffer concentrations above 0·22 per cent sodium bicarbonate depressed the virus yield from 10^7 cells/ml. in the absence of glucose but that higher concentrations of bicarbonate were tolerated (and were necessary for pH control) when glucose was metabolised with rapid acid production. To explain the discrepancy it is necessary to assume that the provision of energy by glycolysis is more sensitive to the " toxic " action of bicarbonate than oxidative processes since oxygen restored the yield of virus from the large culture volumes containing glucose.

For routine virus production in this laboratory, ERK cells have been suspended in ES-G at 10^7/ml. and agitated with a magnetic stirrer. Volumes of cell suspension greater than 1·5 l. have not been tested since they are beyond the present capacity of our cell production ; but there is no reason to suppose that they could not be used. Oxygenation of cell suspensions depends on several variable factors. On the scale used, adequate cell oxygenation was obtained by stirring a relatively shallow layer of suspension under a gas phase of 95 per cent oxygen + 5 per cent CO_2 (v/v) in sealed bottles. The latter point is particularly convenient from a safety aspect. On a larger production scale, however, the use of shallow layers would be cumbersome and uneconomical. By the addition of glucose to the suspending medium it is possible to use a greater depth of cell suspension still within a completely sealed container and therefore obviate the necessity to increase the oxygen solution-rate by continuous gas flow.

To obtain virus yields of 10^{10} PFU/ml. it is necessary to increase the cell concentration to $0{\cdot}5 - 1{\cdot}0 \times 10^8$ cells/ml. The presence of glucose at such high cell concentrations causes difficulty in pH control and the omission of glucose, compensated by adequate cell oxygenation, is therefore convenient. A cell concentration of 10^8/ml. is about the physical limit at which cell suspensions can be made, consisting of about 50 per cent v/v cells.

It should be noted that the measured yield of PFU/cell is dependent on the sensitivity of the virus plaque assay. Our assay system has consistently indicated yields of 100–300 PFU/cell from both monolayer and suspension cultures ; this is in agreement with most reported yields for poliovirus. However, the use of other cells such as " a particularly sensitive clonal strain of HeLa " (Levintow and Darnell, 1960) in the assay might indicate higher yields than those quoted here.

The methods we have described could be applied to the production on a large scale of highly potent virus material suitable as diagnostic antigen, starting

material for chemical investigations and as a vaccine. Although virus produced in serially cultivated cell lines may not be used for human vaccine on the grounds of possible cell malignancy, the use of cells in primary culture overcomes this objection. The production of high titre poliovirus with primary monkey kidney cells will be described elsewhere.

SUMMARY

Poliovirus replicated to high titre (10^9 PFU/ml.) in 10 ml. volumes of 10^7 ERK cells/ml. suspended in a nutrient-free saline and supplied with either oxygen or glucose. Larger cultures (up to 1·5 l.) without glucose were adequately oxygenated by stirring as a shallow layer under a gas phase of 95 per cent oxygen and 5 per cent CO_2 (v/v). These cultures produced high titre poliovirus of Types I, II and III. In a similar way, small volumes of vigorously agitated suspensions containing 10^8 cells/ml. produced 10^{10} PFU/ml. of Type I poliovirus provided that sodium bicarbonate was adjusted to the optical concentration (0·22 to 0·44 per cent for this cell concentration). The addition of glucose to large cultures reduced but did not eliminate the need for oxygenation ; this anomaly is discussed.

The authors wish to thank Mr. J. P. Albericci for growing the ERK cells and Mr. K. Allner, Mr. D. J. Algar and Mr. B. F. Courtney for valuable technical assistance.

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THE INFLUENCE OF ELECTROLYTES ON THE CARDIOVASCULAR EFFECTS OF UNILATERAL RENAL ISCHAEMIA IN RATS*

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Received for publication January 30, 1961

THE decisive role played by electrolytes in the experimental production of myocardial and vascular lesions is well known. It has been shown (Selye, 1958) that the cardiotoxicity of many unrelated agents largely depends on their being simultaneously administered with electrolytes, and, more precisely, on both the cation and anion content of the latter. Certain adrenal steroids that exert both glucocorticoid and mineralocorticoid effects can produce massive cardiac necroses in a great variety of species if, at the same time, the animals are treated with sodium salts of which the anion is either phosphate, sulphate or chlorate (no other sodium-salt is active in this respect). This syndrome was called the electrolyte-steroid cardiopathy with necrosis (ESCN). On the other hand, when given together with sodium chloride to animals suitably sensitized by unilateral nephrectomy, the same steroids can produce a different type of cardiopathy, characterized by hyalinosis, Aschoff nodules and periarteritis nodosa. This pathological entity is referred to as the electrolyte-steroid cardiopathy with hyalinization (ESCH). A similar picture resembling the latter syndrome also accompanies permanent, unilateral, renal ischaemia (Selye, 1946). This condition (the so-called " endocrine kidney syndrome ") was shown, furthermore, to produce not only the ESCH-type of lesions. In a rat bearing an endocrine kidney and treated with disodium phosphate (a sensitizing salt for the ESCN), acute fatal myocardial necroses will ensue instead of the more chronic hyaline and inflammatory changes seen when the animals are not given additional electrolyte treatment (Nádasdi, 1960). This striking similarity between certain corticoid actions and the effects of the endocrine kidney, as regards the production of the ESCN, led us to explore further the possible influence of sodium salts and other electrolytes on the cardiovascular effects of the endocrine kidney. Our findings permitted us first to evaluate the role of cation-anion relationships in the manifestations of unilateral renal ischaemia, and second, to compare the electrolyte dependence of the ischaemic kidney with that of the cardioactive corticoids, as regards cardiotoxicity.

MATERIALS AND METHODS

Three experimental series were performed. In all of them we used female Sprague-Dawley rats from the Quebec Breeding Farm, with an average initial body weight of 146 g. The animals were kept on " Purina Fox Chow® " and tap water *ad libitum*.

* This work was supported by the Gustavus and Louise Pfeiffer Research Foundation, by the National Heart Institute, U.S. Public Health Service (Grant H-3688-C2), and by the National Research Council of Canada (Cons. Grant 11).

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Endocrine kidney operations were performed in accordance with the method described by Selye (1946), by partially ligating the aorta between the two renal arteries, using the stylet of a 22-gauge hypodermic needle. The left ureter was subsequently ligated and transected.

All electrolytes were of the " Reagent " degree of purity and were administered at the dose of 1 mM dissolved in 4 ml. of water twice daily, by stomach tube. The following salts were used: Na_2HPO_4, anhydrous (Fisher), $NaClO_4.H_2O$ (Fisher), Na_2SO_4, anhydrous (Merck), NaCl, anhydrous (Brickman), $NaOOC.CH_3.3H_2O$ (Merck), $Na_3C_6H_5O_7.2H_2O$ (Merck), $Na_2C_4H_4O_6.2H_2O$ (Fisher), $K_2HPO_4.3H_2O$ (Brickman), $Li_3PO_4.1/2H_2O$ (Anachemia), $(NH_4)_2HPO_4$ (Fisher), $MgSO_4.7H_2O$ (Fisher), $KClO_4$ (Fisher), NH_4Cl (Nichols), $MgCl_2.6H_2O$ (Merck), KCl (Fisher), $CaCl_2$ (Fisher).

The operations were performed and treatment was initiated on the first day of the experiment. On the 12th day, the surviving rats were killed with chloroform. The left kidneys were immediately examined to verify the outcome of the operations. Only those rats showing the typical picture of the endocrine kidney were included in the results. Animals with necrotic or hydronephrotic kidneys were discarded. The hearts were examined for macroscopic lesions, but the grading was based only on histological evaluation. For this purpose, the hearts were fixed with Susa solution, embedded in paraffin and stained with the periodic acid Schiff technique. Purely necrotic and periarteritic changes were evaluated separately. Hyaline deposits in the vessels and muscle fibres are not mentioned separately, since under our conditions, they are always concomitant with periarteritis, both changes occurring in the same degree. The severity of the cardiac lesions is expressed in an arbitrary scale of 0 to 3 : 1 designating the smallest muscle fibre destruction or the first oedematous stage of periarteritis in one or two vessels of the heart, to 3, which stands for large, macroscopically visible necrotic foci with intense polymorphonuclear cell reaction and connective tissue invasion, or extensive hyalinosis and periarteritis involving almost all cardiac arteries. Mortality rates, incidence per cent and severity of lesions (together with the standard errors) are shown in the tables.

RESULTS

The cardiotoxic effect of sodium salts plus the endocrine kidney

The effects of the electrolytes can be compared with those seen in Group 1 (Table I), where no treatment was applied other than the endocrine kidney. This

TABLE I.—*The Influence of* Na*-salts on the Cardiac Effect of the Endocrine Kidney*

Group	Treatment*	Number of rats	Cardiac necrosis		Cardiac periarteritis		Mortality (per cent)
			Incidence (per cent)	Severity† (0–3)	Incidence (per cent)	Severity† (0–3)	
1	None	49	43	0·5±0·02	65	1·4±0·14	35
2	Na_2HPO_4	31	93	2·0±0·15	0	0	98
3	$NaClO_4$	17	85	2·1±0·23	0	0	93
4	Na_2SO_4	10	80	1·7±0·32	70	1·4±0·37	40
5	NaCl	14	50	0·6±0·20	94	1·9±0·27	15
6	Na-acetate	17	60	0·8±0·20	72	1·8±0·30	43
7	Na-citrate	20	85	1·9±0·20	55	1·0±0·24	70
8	Na-tartrate	14	57	1·1±0·23	77	1·6±0·28	35

* All salts were given at the dose of 1 mM in 4 ml. of water twice daily during 12 days. In all groups, endocrine kidney operations were performed on the first day of the experiment.

† With the standard errors.

intervention itself produces some necrosis and pronounced periarteritis within 12 days. It is obvious, however, that the same Na-salts that produce necrosis when given together with certain corticoids have an equally necrotizing effect when

combined with the endocrine kidney. Sodium phosphate and sodium chlorate are particularly active in this respect. The complete absence of hyaline and periarteritic lesions in these cases is due to the rapid mortality resulting from cardiac necroses, which ensued in almost 100 per cent of the animals before the slower developing hyalinosis could manifest itself. Sodium sulphate, on the other hand, while producing marked necrosis, did not increase the mortality rate ; thus, the periarteritis had time to develop.

The only Na-salt not active in the ESCN but very cardiotoxic in endocrine-kidney bearing rats was Na-citrate. This salt, like Na_2SO_4, allowed enough time for the development of periarteritis, because of the lower incidence of mortality. The other Na-salts (as in the ESCN) were completely inactive as regards the production of cardiac necrosis.

It was interesting to see that no Na-salt is able to influence the hyalinization and periarteritis that occur following the production of unilateral renal ischaemia.

The cardiotoxic effect of salts other than sodium plus the endocrine kidney

For better statistical evaluation, we tabulated all the non-treated animals conjointly ; therefore, the animals of Group 1, Table I, served as controls for this experiment. As Table II demonstrates, the majority of the non Na-salts were not

TABLE II.—*The Influence of Non-*Na*-salts on the Cardiac Effect of the Endocrine Kidney*

Group	Treatment*	Number of rats	Cardiac necrosis		Cardiac periarteritis		Mortality (per cent)
			Incidence (per cent)	Severity† (0–3)	Incidence (per cent)	Severity† (0–3)	
1	K_2HPO_4	17	44	$0{\cdot}7\pm0{\cdot}22$	73	$1{\cdot}3\pm0{\cdot}3$	67
2	Li_3PO_4	10	60	$0{\cdot}7\pm0{\cdot}20$	0	0	100
3	$(NH_4)_2HPO_4$	8	40	$0{\cdot}6\pm0{\cdot}36$	85	$2{\cdot}1\pm0{\cdot}36$	40
4	$MgSO_4$	16	69	$0{\cdot}7\pm0{\cdot}14$	75	$1{\cdot}8\pm0{\cdot}32$	56
5	$KClO_4$	17	76	$1{\cdot}1\pm0{\cdot}18$	30	$0{\cdot}4\pm0{\cdot}18$	94
6	NH_4Cl	17	42	$0{\cdot}5\pm0{\cdot}17$	54	$1{\cdot}0\pm0{\cdot}26$	28
7	$MgCl_2$	14	30	$0{\cdot}3\pm0{\cdot}14$	36	$0{\cdot}7\pm0{\cdot}30$	70
8	KCl	9	33	$0{\cdot}4\pm0{\cdot}25$	56	$1{\cdot}2\pm0{\cdot}40$	66
9	$CaCl_2$	20	80	$1{\cdot}3\pm0{\cdot}20$	60	$1{\cdot}1\pm0{\cdot}26$	80

* All salts were given at 1 mM dose in 4 ml. of water twice daily during 12 days. In all groups, endocrine kidney operations were performed on the first day of the experiment.

† With standard errors.

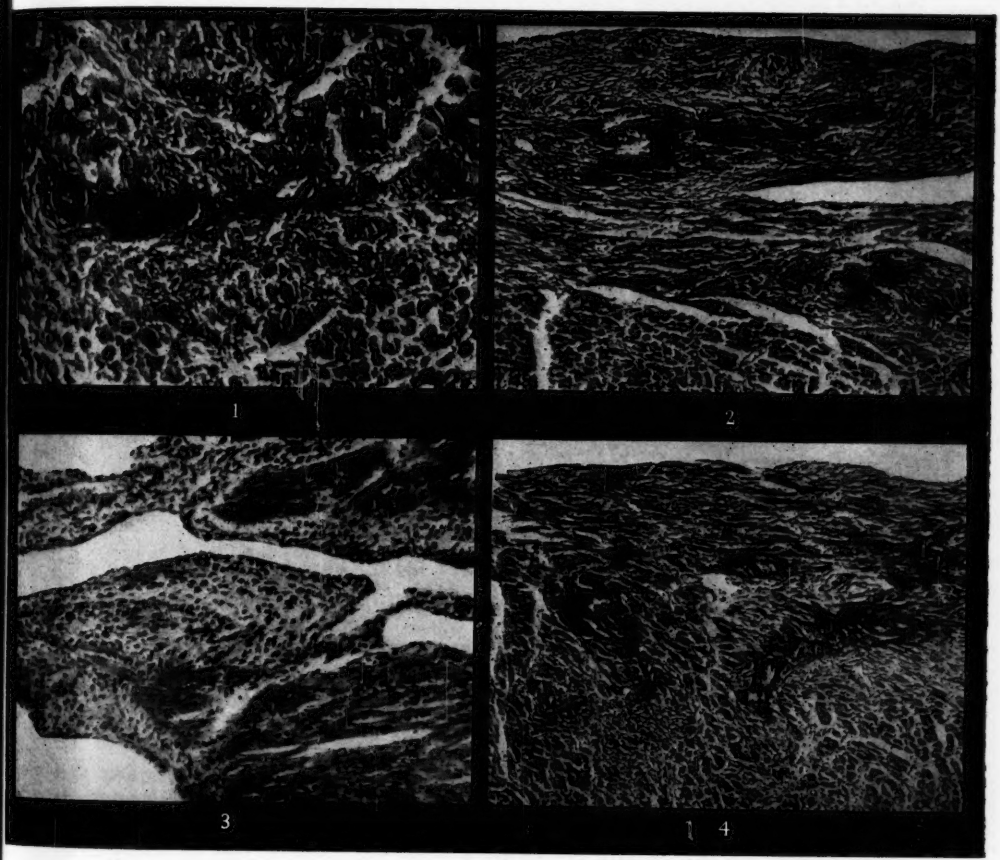
EXPLANATION OF PLATE

FIG. 1.—Hyalinization of coronary arteries of a rat bearing an " endocrine kidney ". The massive hyaline deposition completely obstructs the vessel. The dark areas correspond to the red, PAS-positive material. PAS. ×124.

FIG. 2.—Hyalinization and degeneration of the myocardium of a rat bearing an " endocrine kidney ". The dark spots represent the deposition of the PAS-positive hyaline material, surrounded by a halo of degenerative muscle fibres. PAS. ×83.

FIG. 3.—Myocardial necrosis in a rat bearing an " endocrine kidney " and treated with Na_2HPO_4. Note the sub-endocardial and septal predilection and the destruction of a papillary muscle. PAS. ×124.

FIG. 4.—Conjoint hyalinization and necrosis in the heart of a rat bearing an " endocrine kidney " and treated with Na-citrate. PAS. ×83.



cardiotoxic. This result is identical with that seen for the ESCN. Unlike in the ESCN, $KClO_4$ and $CaCl_2$ can significantly increase the occurrence and severity of cardiac necroses when given to rats bearing an endocrine kidney. In both cases, mortality was high ; but in the group treated with $CaCl_2$ the incidence of periarteritis was similar to that of the controls. Potassium chlorate did not permit the full development of the arterial lesions, owing to the early death of almost all animals.

We did not deem it necessary to include a control, non-endocrine kidney-bearing group treated with electrolytes in these 2 experiments, as previous extensive studies (Selye, 1958) had already clearly proved that in intact, otherwise untreated animals, each of these salts is devoid of any cardiovascular effect.

Attempted prevention of cardiac necroses produced by Na_2HPO_4 *plus the endocrine kidney, using* KCl, $MgCl_2$ *or* NH_4Cl

Since it was known that KCl and $MgCl_2$ completely protect the heart against the manifestations of the ESCN, and that NH_4Cl acts similarly against the ESCH, it was important to investigate whether these same salts could also protect against the respective lesions when unilateral renal ischaemia is substituted for the corticoids. Much to our surprise, despite the similarities between the ESCN and the lesions produced as outlined in the preceding experiments, KCl, $MgCl_2$, and NH_4Cl had no protective effect in our experimental arrangement. (Table III.) In fact,

TABLE III.—*Attempted Prevention of Cardiovascular Lesions by* KCl, $MgCl_2$ *or* NH_4Cl

Group	Treatment*	Number of rats	Cardiac necrosis		Cardiac periarteritis		Mortality (per cent)
			Incidence (per cent)	Severity† (0–3)	Incidence (per cent)	Severity† (0–3)	
1	H_2O	8	63	1·1±0·34	12	0·2±0·24	100
2	KCl	9	33	1·7±0·37	11	0·3±0·35	100
3	$MgCl_2$	9	66	1·0±0·29	66	1·7±0·47	73
4	NH_4Cl	7	57	0·8±0·34	44	0·6±0·29	100

* In addition, all the rats received Na_2HPO_4 as indicated in the text and endocrine kidney operations were performed in all groups on the first day of the experiment.
† With standard errors.

KCl was much more toxic in the endocrine-kidney group than in intact rats as is evidenced by the finding that almost all animals died on the second day of treatment. This explains also the relatively low incidence of both necrosis and periarteritis in this group. The same explanation applies for the group treated with NH_4Cl in which all rats died within 5 days. Magnesium chloride did not increase toxicity or exert a protective action on the heart.

DISCUSSION

Our experiments on the production of cardiac necrosis revealed some striking similarities between the effect of certain corticoids and that of the endocrine kidney. In both cases the same sodium salts, namely phosphate, sulphate and chlorate, are essential for sensitizing the heart. There was one exception to this however ;

sodium-citrate when combined with the endocrine kidney also produces massive myocardial necroses. Bajusz and Selye (1960) have shown that certain otherwise innocuous sodium salts, *e.g.*, sodium-citrate, might have a necrotizing effect when given to animals kept on a low chloride diet. Schaffenburg and Selye (1948) had earlier found that the endocrine kidney produces marked hypochloraemia. These two facts may explain the behaviour of sodium-citrate in our experiment.

It should be pointed out that not all sodium-salts are equally effective in the cardiopathies elicited by the endocrine kidney. This is important if one considers the equivocal data in the literature, claiming the significance of the sodium-ion *per se* in the development and maintenance of renal hypertension and cardiovascular toxicity. There are some indications that it is the cation-anion relationship that influences renal cardiovascular effects. Selye, Stone, Timiras and Schaffenburg (1949) prevented the cardiotoxic effect of deoxycorticosterone in rats, by maintaining the animals on a sodium- and chloride-free diet. Danford and Herrin (1952) obtained the same dietary result in renal hypertensive rats. Lehr, Churg and Milora (1953) found that a diet free from or deficient in sodium alone does not prevent the cardiac damage and hypertension produced by renal injury in the same species. Martini and Kaiser (1954) confirmed this in human subjects and these authors stated that, for the induction of hypertension, sodium and chloride must be given conjointly, as neither is effective alone.

The inability of sodium to influence the hyaline and periarteritic changes produced by the presence of an endocrine kidney was known from the experiments of Selye and Stone (1948). Our findings further demonstrate that even varying the anion combinations does not enable sodium to aggravate these lesions. This is more interesting if we consider that similar cardiovascular damage resulting from mineralocorticoid overdosage in the rat is strictly dependent on simultaneous sodium chloride administration (Selye *et al.*, 1949). It has been shown, furthermore, that the mineralocorticoid effect can also be aggravated by other sodium salts, such as sodium bicarbonate or sodium sulphate (Selye, Mintzberg and Rowley, 1945*b*). A comparison between these data and our present results suggests that the endocrine kidney affects the impairment of electrolyte metabolism more profoundly than do the mineralocorticoids, which apparently depend on additional exogenous sodium. This fact might explain why the hypertensive action of the endocrine kidney is not further aggravated by additional sodium salts. This hypertensive action, however, must be clearly differentiated from the necrotizing effect of such a kidney, which, as we have seen, greatly depends on the anionic combination of the applied sodium salts. Here again, we refer to the work of Selye *et al.* (1945*b*), who observed that sodium sulphate (a salt that greatly sensitizes the heart for the production of necrosis by the endocrine kidney) does not have this effect when the cardiotoxic agent is a mineralocorticoid and not the endocrine kidney.

The inactivity of the non-sodium salts in our experiment is similar to that seen in the ESCN. In the case of the endocrine kidney, however, two salts, potassium chlorate and calcium chloride were exceptions. The suppression exerted by the former compound on thyroid activity suggested that there may exist an interaction between the endocrine kidney and the thyroid. Experiments are now being performed to investigate this possibility. The cardiac necrosis obtained with calcium chloride in the endocrine-kidney-bearing rats again offers some interesting comparisons with the cardiac effect of mineralocorticoids. In

the latter case, calcium chloride gave the most complete protection against both hyalinosis and periarteritis (Selye *et al.*, 1945*b*). In the former group, however, this salt was not only devoid of any protective action, but it produced marked myocardial necrosis. Experiments are under way to see the effect of calcium salts other than chloride when combined with the endocrine kidney.

The observation that none of the salts that proved to be protective in either the ESCN or the ESCH had any beneficial effect in our experiment was one of the most important results. It indicates that, while the manifestations of electrolyte plus corticoid treatment are identical to those of electrolyte administration plus renal ischaemia, there must be some difference between the two pathways by which these agents exert their effects. This becomes even more significant if we consider that potassium chloride and magnesium chloride proved to be fully protective in all those necrotizing cardiopathies that can be produced or aggravated by the sensitizing sodium salts. Ammonium chloride, furthermore, gives complete prevention against the hyalinizing type of cardiopathy produced by mineralocorticoids plus sodium chloride (Selye, Hall and Rowley, 1945*a*). The same lesions, nevertheless, when produced by the endocrine kidney, are resistant to treatment with this salt. The fact that potassium chloride and magnesium chloride did not protect against endocrine-kidney-induced lesions corresponds to the findings obtained with mineralocorticoid-induced lesions (Selye *et al.*, 1945*b*).

We have not so far found any compound that offers protection against the cardiovascular effects of the endocrine kidney, whether this intervention is alone or in combination with sensitizing electrolytes.

Finally, it should be emphasized that the manifestations obtained with unilateral renal ischaemia plus certain salts are not mere consequences of the partial lack of renal excretory function. This was clearly demonstrated in some preliminary experiments in which neither unilateral nephrectomy (even when applied conjointly with severe stress) nor the removal of a pre-existing endocrine kidney, plus the cardiotoxic sodium salts, were capable of producing the cardiac lesions readily seen when the non-excreting ischaemic kidney is retained in the organism. Thus, the substance(s) of an ischaemic kidney (the effect of which is very similar to that of mineralocorticoids) has a definite active role in the production of the metabolic derangement that ultimately leads to fatal cardiovascular lesions.

SUMMARY

When certain sodium salts, such as Na_2HPO_4, Na_2SO_4, or $NaClO_4$ are given to rats bearing a so-called "endocrine kidney" massive and often fatal cardiac necroses occur. The same picture had previously been observed when instead of being subjected to the kidney intervention the animals were given steroids conjointly with the sodium salts. In both cases, the anion attached determines the cardiotoxic effect of sodium. None of the sodium salts tested had any influence upon the myocardial hyalinization or periarteritis nodosa normally seen as a result of the endocrine kidney itself.

Most of the non-sodium salts, with the exception of $KClO_4$ and $CaCl_2$, are devoid of cardiotoxicity when applied conjointly with the endocrine kidney.

The compounds known to protect the heart against the combined effect of electrolytes and steroids (namely, KCl, $MgCl_2$, and NH_4Cl) are completely inactive if the cardiac lesions are produced by the endocrine kidney plus electrolytes. This

indicates that, while the end result in both cases is identical, there must be a basic difference between the mechanisms through which the endocrine kidney and the corticoid hormones exert their cardiotoxic effects.

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THE HEXOSAMINE CONTENT OF THE KIDNEY IN EXPERIMENTAL RENAL CALCIFICATION

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Received for publication February 1, 1961

RECENT studies on experimental animals have led to the conclusion that mucopolysaccharides, which appear to play an important role in the calcification of cartilage, may also be intimately involved in pathological calcification of the kidney and the aorta.

Rubin and Howard (1950) observed a marked increase in the staining intensity of the ground substance in regions of active calcification in young bones and attributed this to either an increase in the amount of mucopolysaccharides present or a change in their degree of polymerization. Dziewiatkowski, Benesch and Benesch (1949) observed that on injection of sulphate containing ^{35}S into young rats, the tracer was preferentially retained in regions of active calcification, and Castellani and Zambotti (1956) found that homogenates of epiphyseal plate of growing rabbits could synthesize greater amounts of hexosamine than any other tissue. These results suggest that calcification of cartilage involves a synthesis of mucopolysaccharides.

An increase in the staining intensity of the mucopolysaccharides of the ground substance has been observed in human atherosclerotic lesions by Faber (1949) and in calcified rat kidney by Baker and Sison (1954) and Grimes (1957). Buck and Heagy (1958) found that rabbit aortae which had developed atheromatous plaques as a result of cholesterol feeding took up more ^{35}S than normal aortae and an increased uptake of ^{35}S has been observed in the calcifying kidney by Bradford, Howard, Joel and Shetlar (1959) and Reaven, Schneider and Reaven (1960). This evidence suggests that calcification in the aorta and in the kidney also involves a synthesis of mucopolysaccharides, a hypothesis which is strengthened by the recent observation of Gillman, Grant and Hathorn (1960) that the deposition of calcium and phosphorus in the aortae of calciferol-fed rats is accompanied by a 100 per cent increase in the total hexosamine present.

In the present investigation further information has been sought regarding the mechanism of calcification in the kidney by comparing the hexosamine contents of normal and calcifying rat kidneys, hexosamine being taken as an index of the amount of mucopolysaccharides present.

MATERIALS AND METHODS

Production of calcification in the rat kidney

Male, Wistar Albino rats, weighing 100–200 g. were given free access to water and food consisting of nuts of "Diet 41B" obtained from E. Dixon and Sons, Ltd., Middlesex; calcification of the kidney did not occur spontaneously on this diet. Paired rats of similar

initial weight were used, one rat of each pair acting as a control while the other received NaH_2PO_4, vitamin D_2 or parathyroid extract. The following methods of producing calcification were used :—

Experiments 1(*a*) *and* 1(*b*). *Sodium dihydrogen phosphate.*—The test animals received 2 ml. of NaH_2PO_4 solution, twice daily, 6 days a week, by polythene stomach tube under light ether anaesthesia. In experiment 1(*a*) the test animals received 15 per cent NaH_2PO_4 solution for 2–4 weeks. In experiment 1(*b*) the test animals received 10 per cent NaH_2PO_4 for 2 days followed by 15 per cent NaH_2PO_4 for 2 weeks and finally 20 per cent NaH_2PO_4 for 1 week All the control animals received 2 ml. of water by stomach tube whenever the corresponding test animals received phosphate solution.

Experiment 2. *Vitamin* D_2.—The test rats received 1 ml. of " Radiostol " (obtained from British Drug Houses, Ltd. and containing 180,000 I.U. of vitamin D_2 per ml.) subcutaneously on alternate days. The duration of the experiment was varied to suit the health and general response to treatment of each rat and the rats received total doses varying from 720,000 to 1,620,000 I.U. The control animals received 1 ml. of arachis oil subcutaneously whenever the corresponding test animals received " Radiostol ".

Experiment 3. *Parathyroid Extract.*—The test rats received daily intraperitoneal injections of " Parathormone " (obtained from Eli Lilly and Co. Ltd. and containing 100 U.S.P. units per ml.) 6 days a week for a period of 1–2 weeks, in gradually increasing doses as recommended by Chown, Lee, Teal and Currie (1939). The dose was varied to suit the response and general state of each animal and the total doses administered varied from 1,100 to 1,700 U.S.P. units. " Parathormone " contains 0·2 to 0·3 per cent phenol as a preservative and the control animals were therefore given an equal volume of 0·25 per cent phenol in physiological saline intraperitoneally whenever the corresponding test animals received " Parathormone ".

Analysis of kidney tissue

Kidney homogenates were analysed for hexosamine (as an index of the amount of mucopolysaccharides present), calcium, inorganic phosphorus and nitrogen. Erroneously high values for kidney inorganic phosphorus were obtained unless kidney alkaline phosphatase activity was inhibited during removal and homogenization of the kidneys and the following procedure was therefore adopted.

The rat was killed with ether and 20 ml. of ice-cold physiological saline perfused into the left ventricle of the heart. The inferior vena cava was cut just below the diaphragm and the kidneys, which were pale in colour following exsanguination, were removed, washed with ice-cold saline, and the kidney capsules dissected away together with any adhering fat. The kidneys were blotted dry, weighed, and one kidney was cut longitudinally. One half kidney was fixed in formol saline for histological examination and the remaining one and a half kidneys were re-weighed and homogenized in 4–5 ml. of ice cold 0·025N HCl in a cold all-glass homogenizer of the Potter-Elvehjem type. The homogenate was transferred quantitatively to a cold 25 ml. graduated tube and diluted to a suitable volume with cold 0·025N HCl so that the final concentration was approximately 10 per cent. The whole procedure from the killing of the animal was carried out as rapidly as possible.

Inorganic phosphorus.—Immediately after homogenization, a 1 ml. sample of the homogenate was extracted with three 5 ml. aliquots of 10 per cent trichloroacetic acid. The extracts were combined, made up to 20 ml. with 10 per cent trichloroacetic acid and filtered. The inorganic phosphorus content of the filtrate was determined by a modification of the method of Berenblum and Chain (1938) in which the colour was developed by the addition of 0·1 ml. of a 40 per cent solution of stannous chloride in concentrated HCl (Bartley, 1953).

Calcium.—A suitable volume of homogenate (4–6 ml. from a control animal or 2–3 ml. from an experimental animal) was placed in a 15 ml. conical Pyrex centrifuge tube and maintained at 80° for several days until dry. The residue was ashed at 500° for 6 hr. in a muffle furnace, 1 ml. of 1N HCl was added and the contents dried and ashed again as described previously. To the white ash was added 1 ml. of 0·1N HCl, the tube was placed in a boiling water bath for 15 min. and the contents stirred with a fine glass rod. The acid solution was cooled and its calcium content determined by a modification of the method of Clark and Collip (1925) in which the calcium was precipitated by the addition of 2 ml. of buffered ammonium oxalate solution (Levine and Cummings, 1956).

Nitrogen.—The nitrogen content of the homogenate was determined on 1 ml. samples by the micro-Kjeldahl method (Markham, 1942).

Hexosamine.—The homogenate (2 ml.) was hydrolysed with 2 ml. of 8N HCl in a stoppered tube at 100° for 4 hr. After diluting to 10 ml. with water, the mixture was filtered and 5 ml. of filtrate was evaporated to dryness at room temperature over $CaCl_2$ and NaOH *in vacuo*. The residue was dissolved in water, the residual acid neutralized with dilute NaOH using phenolphthalein as indicator and the hexosamine content determined by the method of Rondle and Morgan (1955) as modified by Kraan and Muir (1957). The recovery of hexosamine from purified chondroitin sulphate was 86 per cent. According to Partridge and Davis (1958), 10–14 per cent of galactosamine is destroyed on heating in 4N HCl for 16 hr. and the recovery of hexosamine from chondroitin sulphate was therefore considered to be satisfactory. Removal of interfering chromogens by preliminary treatment of the hydrolysate with a cation exchange resin (Dowex 50 as recommended by Boas, 1953, or Amberlite IRC-50 as recommended by Exley, 1957) was found to be unnecessary in the case of rat kidney tissue.

RESULTS

General observations

The response to treatment varied but the majority of the test animals either lost weight or gained less than the corresponding controls. The rats tolerated phosphate treatment well, whereas dosage with vitamin D_2 and parathyroid extract caused toxic symptoms. In the final stages the latter agents caused marked loss of appetite and weight, the limb bones became thin and the animals were listless and had difficulty in moving.

When the total kidney weight was expressed as a percentage of the final body weight, the values obtained for calcified kidneys were greater than those obtained for normal kidneys. This difference was due in part to an increase in the size and weight of the kidneys of the test animals and in part to the reduced rate of growth of the test animals compared with the controls.

Macroscopic findings

On gross examination the surface of the kidneys of rats treated with vitamin D_2 or parathyroid extract had a white, mottled appearance. Longitudinal sections, of the kidneys obtained by the administration of each of the three agents frequently revealed the presence of a distinct white band of calcification, visible to the naked eye, which was located roughly in the region of the cortico-medullary junction. Treatment with parathyroid extract also caused the appearance of white striations which radiated inwards from the surface through the cortex.

Microscopic findings

Sections of the calcified kidneys were stained with haematoxylin and eosin or the von Kossa stain and it was observed that the kidneys from phosphate-treated rats bore the closest resemblance in their histological appearance to the human material removed in cases of nephrocalcinosis. In these kidneys the calcium deposits were granular and were found in the cortico-medullary boundary zone. In the kidneys of rats treated with vitamin D_2 or parathyroid extract the deposits were larger and the renal damage was more extensive. The calcification produced by vitamin D_2 occurred chiefly in the inner cortex whereas that caused by parathyroid extract had a wider distribution and was found throughout the cortex.

Chemical findings

The degree of calcification induced by each agent varied considerably from one rat to the next and parathyroid extract was the most effective of the three (Table

I). The total nitrogen content of the calcified kidneys was generally greater than that of normal kidneys (Table II). When expressed in terms of the wet weight of the tissue, however, the nitrogen content of the calcified kidneys tended to be lower than that of normal kidneys. The tubules of the calcified kidneys were further apart and the kidneys appeared to be oedematous. The increase in weight of the

TABLE I.—*Mean Values for the Calcium and Inorganic Phosphorus Contents of Normal and Calcified Kidneys**

Experiment	Agent administered	Number of animals	Calcium (mg. per g. wet weight)	Inorganic phosphorus (mg. per g. wet weight)
Control	None	17	0·063 (0·048–0·086)	0·39 (0.35–0.49)
1 (*a*)	Sodium dihydrogen phosphate	6	0·55 (0·19–1·49)	0·82 (0·45–1·58)
1 (*b*)	Ditto	5	0·71 (0·19–1·10)	0·78 (0·51–0·99)
2	Vitamin D_2	4	1·00 (0·11–1·90)	0·78 (0·48–1·32)
3	Parathyroid extract	4	2·06 (0·49–4·41)	1·52 (0·62–3·09)

* Figures in brackets show the range of values observed.

TABLE II.—*Mean Values for the Nitrogen and Hexosamine Contents of Normal and Calcified Kidneys**

Experiment	Agent administered	Number of animals	Total kidney nitrogen (mg.)	Nitrogen (mg. per g. wet weight)	Total kidney hexosamine (mg.)	Hexosamine (mg. per g. wet weight)	Hexosamine (mg. per g. of nitrogen)
1 (*a*)	None (control)	6	40·9 (29·2–49·1)	24·5 (22·8–25·5)	2·52 (1·82–3·09)	1·60 (1·37–1·72)	62·2 (54·4–69·3)
1 (*a*)	Phosphate	6	56·1 (51·7–58·9)	22·5 (22·2–23·2)	3·57 (2·62–4·65)	1·40 (1·31–1·53)	62·9 (59·0–65·9)
1 (*b*)	None (control)	5	36·1 (30·6–39·8)	25·8 (24·4–27·0)	2·35 (2·03–2·51)	1·68 (1·56–1·82)	64·9 (61·9–67·4)
1 (*b*)	Phosphate	5	41·4 (34·9–50·8)	22·4 (17·4–24·9)	2·82 (2·30–3·80)	1·51 (1·30–1·64)	67·8 (62·5–74·7)
2	None (control)	2	32·5 (32·4 and 32·5)	26·1 (25·9 and 26·4)	2·02 (2·02 and 2·02)	1·65 (1·64 and 1·65)	62·9 (62·1 and 63·7)
2	Vitamin D_2	4	26·5 (23·2–32·5)	21·8 (19·6–26·2)	1·74 (1·59–2·12)	1·43 (1·27–1·71)	65·5 (61·9–69·4)
3	None (control)	4	27·9 (23·4–30·6)	24·8 (23·8–26·3)	1·77 (1·49–1·98)	1·57 (1·48–1·67)	63·4 (60·9–65·5)
3	Parathyroid extract	4	38·2 (28·3–45·1)	20·3 (17·5–24·9)	2·48 (1·62–3·30)	1·29 (1·16–1·37)	64·2 (55·0–73·2)

* Figures in brackets show the range of values observed.

calcified kidneys was therefore due partly to the accumulation of water and partly to hypertrophy of the kidney tissue.

The kidney hexosamine content was expressed in three ways (Table II). When expressed as total kidney hexosamine, the values obtained for calcified kidneys were frequently greater than those for normal kidneys, whereas there was a decrease in the amount of hexosamine per g. wet weight of kidney during calcification. Finally, when the hexosamine was expressed as mg. per g. of kidney nitrogen, there was no difference between the hexosamine contents of normal and calcified kidneys and this appears to be the most satisfactory means of expression for reasons which will be discussed later.

DISCUSSION

The role of mucopolysaccharides in the formation of atheromatous plaques had been extensively investigated (Faber, 1949; Taylor, 1953; Buck 1955; Buck and Heagy, 1958; Schwartz, Peters and Day, 1958). Gillman *et al.* (1960) compared the chemical and histochemical changes occurring in calciferol-induced vascular lesions. These authors observed that the administration of calciferol caused a heavy deposition of calcium and phosphorus in the aorta and a 2–3-fold increase in the mean dry weight of the aorta. The total aortic hexosamine of calciferol-treated rats was twice that of control rats and the authors concluded that since the hexosamine content of the aorta had increased, the histochemical changes which had indicated an increased concentration of mucopolysaccharides were, in fact, a reliable indication of the chemical changes occurring. When the aortic hexosamine was expressed as a percentage of the dry fat-free weight, however, the values obtained for calciferol-treated and control rats were the same. The authors do not discuss this observation but base their conclusions on the increase in total aortic hexosamine.

In the present investigation, calcification of the kidney was generally accompanied by a rise in the total kidney hexosamine. This increase could be the result of either a specific increase in the amount of hexosamine present during calcification or a general hypertrophy of the kidney. In terms of the wet weight of kidney, however, calcification was accompanied by a decrease in the amount of hexosamine present and this decrease appeared to be the result of dilution due to the deposition of calcium salts and the accumulation of water.

Of the three methods of expressing hexosamine which we have considered, the most satisfactory appears to be in terms of the nitrogen content of the kidney, since this eliminates errors due to both dilution and hypertrophy. When expressed in this way there was no significant difference between the hexosamine contents of normal and calcified kidneys.

Recent studies on the metabolism of hexosamine in calcifying cartilage suggest that an increased synthesis of mucopolysaccharides is not necessarily accompanied by an accumulation of the material synthesized and that the general picture is one of increased turnover. Dikshit (1959) studying the synthesis of hexosamine by rachitic epiphyseal plate before and after treatment with vitamin D, observed that therapeutic doses of vitamin D caused the hexosamine synthesizing capacity of the rachitic plate to increase by a factor of 1·7. There was, however, no corresponding increase in the total hexosamine content of the healing plate but on the contrary, a slight decrease.

In a study of the sulphate metabolism of cartilage, Coelho and Chrisman (1960) found, in confirmation of the results of other workers, that ^{35}S was taken up by cartilage slices when the latter were incubated in a suitable medium containing radioactive sulphate. There was also a loss of total sulphate into the medium, however, during incubation. The amount of ^{35}S taken up was small compared with the loss of sulphate and the authors concluded that the true picture of metabolic activity under these conditions was one of degradation rather than synthesis.

A similar situation may exist in the calcifying kidney, namely, an increased turnover of mucopolysaccharides without any accumulation of the material synthesized.

SUMMARY

A comparison of the hexosamine contents of normal and calcifying rat kidneys has been made in an attempt to elucidate the mechanism of calcification in the kidney.

The total hexosamine content of the calcified kidney was frequently greater than that of the corresponding control. The interpretation of changes in kidney hexosamine content, however, is complicated by accompanying changes in the water content of the tissue and by hypertrophy of the calcifying kidney. The most satisfactory method of expressing the kidney hexosamine content appears to be in terms of tissue nitrogen and when expressed in this way there was no significant difference between the values observed for normal and calcified kidneys.

The significance of this result is considered in the light of recent work on the calcification of cartilage.

We wish to thank Dr. C. K. Anderson for the histological studies, Dr. Helen Muir for a sample of chondroitin sulphate and Eli Lilly and Co. Ltd., for a gift of " Parathormone ".

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HEPATIC GRANULOMAS INDUCED IN GUINEA-PIGS BY FREUND'S ADJUVANT WITH AND WITHOUT HOMOLOGOUS LIVER*

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Received for publication February 7, 1961

THE nature and pathogenesis of the hepatic lesions produced in guinea-pigs by (*a*) Freund's adjuvant and (*b*) Freund's adjuvant with homologous liver was studied. This experiment was prompted by two considerations: (1) Behar and Tal (1959), Chase (1959), Laufer, Tal and Behar (1959), Heymann, Hachel and Hunter (1960), Pearson (1959) have reported hepatic granulomas as part of a general stimulation of the reticulo-endothelial system (Rupp, Moore and Thoenberg, 1960) by Freund's adjuvant. Their studies were performed several weeks after the first injection and did not clarify the development and pathogenesis of the hepatic granulomas, nor their relation to the foci of hepatic necrosis that were sometimes found (Pearson, 1959; Behar and Tal, 1959; Steiner, Langer, Schatz and Volpe, 1960). (2) In spite of the possible importance of auto-immune processes in liver disease only one group (Behar and Tal, 1959) attempted to produce experimental liver disease with Freund's adjuvant and homologous liver but the early occurrence of their lesions speaks against hypersensitivity.

MATERIALS AND METHODS

The experiments were performed on 164 mixed stock female guinea-pigs (Rockland Farms, New City, N.Y.) weighing 250–400 g. and fed Rockland farm diet. All animals received a single injection, deep in the neck muscles, of 2 ml. of 1 of the 4 mixtures described below. Mixture 1 was Difco's complete Freund's adjuvant (composition: 8·5 ml. Bayol F, 1·5 ml. Arlacel, *Mycobacterium butyricum* 5 mg./10 ml.). Mixture 2 was a 20 per cent suspension (wt./vol.) of normal guinea-pig liver in Freund's adjuvant, which was homogenized in a Waring Blendor, quick frozen at − 30° and thawed immediately before use. Mixture 3 was prepared as mixture 2 except that normal liver was replaced by the liver of guinea-pigs which had been injected intraperitoneally with 0·3 m.l. per kg. of CCl_4 diluted 1 to 5 parts in sterile mineral oil and killed 48 hr. later; these livers showed characteristic centrolobular necrosis. Mixture 4 was prepared as mixture 2 except that physiological saline was used instead of Freund's adjuvant. Sterility tests were performed on all mixtures before use. Groups of 20–36 guinea-pigs were killed 3 weeks after the injection and, to study the development of the lesions, groups of 4–12 guinea-pigs were killed at 4–60 day intervals after the injection (Table I). The guinea-pigs were killed by ether anaesthesia and a specimen of liver was removed for cultures on a blood plate, Littman's medium, Petragnani's medium and glucose and thioglycollate broths. These cultures were negative except in 3 instances which were not included in the observations. Abdominal, thoracic organs and the brain were examined grossly. After formalin fixation, one section was cut of: spleen, pancreas, small intestine, paracaecal lymph node, adrenal, kidney, heart, brain and site of injection. Five sections were cut of liver and 2 of lungs. In addition to H. and E. stains, in selected instances, oil red O, PAS, acid fast and azure blue stains and silver impregnations were performed.

* Supported in part by Grant 2G-115, National Institutes of Health, Public Health Service (Graduate Training Grant in Pathology).

D. K. is a Clinical Trainee, supported by the National Cancer Institute, Grant CLT-7026.

TABLE I.—*Plan of Experiment*

Mixture injected	Time after injection when guinea-pigs were killed	Number of guinea-pigs
1	3 weeks	36
2	,, ,,	23
3	,, ,,	20
4	,, ,,	30
2	4 days	9
1	11 ,,	12
2	35 ,,	4
1	35 ,,	6
2	60 ,,	8
None	—	16

RESULTS

Liver lesions in controls (untreated and given mixture 4).—Some of the control livers showed a moderate lymphocytic infiltration of the portal triads, a variable degree of prominence of the Kupffer cells, occasional small necrotic foci without inflammatory infiltration in about every 10th section and very rare necrotic foci surrounded by a rim of segmented leucocytes.

Lesions 3 *weeks after injection of mixtures* 1, 2 *and* 3.—The alterations following the injection of the 3 mixtures were identical in nature and distribution and varied only quantitatively. In the liver, the major feature was multiple granulomas (Fig. 1), located randomly throughout the lobules, measuring up to 300 μ and composed predominantly of epithelioid cells (Fig. 2). Giant cells (Fig. 3) and plasma cells were less frequent. Scattered necrotic liver cells and lymphocytes, histiocytes and segmented leucocytes were found in some granulomas (Fig. 4). Caseation was absent. Within the granulomas, the reticulum network appeared preserved (Fig. 5). Necrotic liver cells within the granulomas and, even more so, adjacent to the granulomas contained fine fat droplets ; the epithelioid cells showed no oil red O staining material. The granulomas contained no PAS positive or acid fast material nor bacterial bodies. The remainder of the hepatic parenchyma showed occasional acidophilic necrotic foci with about the same frequency as the controls. There was no massive necrosis. The Kupffer cells were often prominent. The portal triads showed an increased infiltration with plasma cells and lymphocytes. The bile ducts and blood vessels were unremarkable. The lungs showed multiple subpleural or peribronchial granulomas composed mainly of epithelioid and plasma cells and, in some guinea-pigs, a diffuse infiltration of the alveoli with histiocytes. The spleen had multiple granulomas (Fig. 6), conspicuous diffuse reticulo-endothelial hyperplasia and, in a few cases, extensive haematopoiesis. The lymph nodes exhibited reticulo-endothelial hyperplasia and prominent germinal centres. The other organs were unremarkable except for rare perivascular cuffing with lymphocytes in the myocardium and mononuclear infiltrates in the renal pelvis.

Counts of hepatic granulomas 3 *weeks after injection with mixtures* 1, 2 *and* 3.—The counts were performed on the livers of 36 guinea-pigs injected with Freund's adjuvant alone (mixture 1), 23 guinea-pigs injected with Freund's adjuvant plus homologous liver (mixture 2) and 20 guinea-pigs injected with Freund's adjuvant plus homologous necrotic liver (mixture 3). All groups were killed 3 weeks after injection. In each liver, the numbers of granulomas in 30 low power fields ($\times 100$)

TABLE II.—*Counts of Granulomas in Livers of Guinea-pigs 3 Weeks After Injection of Freund's Adjuvant With or Without Homologous Liver*

Material injected	Number of guinea-pigs	Number of granulomas
Mixture 1	36	1, 2, 3, 3, 4, 4, 4, 4, 5, 5, 5, 6, 6, 7, 7, 8, 8, 9, 11, 12, 13, 13, 14, 14, 15, 18, 18, 19, 20, 27, 30, 40, 53, 61, 137, 174.
Mixture 2	23	2, 3, 6, 8, 9, 9, 11, 12, 15, 16, 16, 16, 21, 22, 27, 27, 33, 43, 47, 95, 111, 410, 603.
Mixture 3	20	0, 1, 3, 5, 8, 10, 10, 14, 15, 15, 20, 25, 33, 39, 73, 79, 126, 139, 271, 324.

Each number represents the number of granulomas counted in 30 low power fields in one guinea-pig liver.
The numbers have been arranged in ascending order.

were counted (Table II). In comparing the frequency of granulomas in the presence or absence of liver in the Freund's adjuvant mixture, the groups given mixtures 2 and 3 were combined. The median number of granulomas in the guinea-pigs that received Freund's adjuvant alone was 10 and in the guinea-pigs that received Freund's adjuvant plus liver it was 16. This difference is significant at the 5 per cent level as shown by the median test. This statistical analysis was performed with the help of the Division of Biostatistics, School of Public Health and Administrative Medicine, Columbia University.

Liver lesions at varied intervals after injection with mixtures 1, 2 and 3.—Guinea-pigs killed 4 days after injection with mixture 2 showed numerous scattered foci composed of small groups of necrotic liver cells, few histiocytes and occasional polypmorphonuclears (Fig. 7). These small foci were surrounded by a rim of fat containing hepatic cells. At 11 days following injection with mixture 1, there were small necrotic foci with some epithelioid cells. At 35 and 60 days after injection the hepatic lesions were not different from the ones found at 3 weeks; few of the granulomas contained necrotic liver cells and segmented leucocytes. No evidence of healing of the granulomas or of fibrosis was noted.

DISCUSSION

The experiments indicate that intramuscular injection of Freund's adjuvant induces only one type of focal hepatic lesion, with a predominance of necrosis at

EXPLANATION OF PLATES

FIG. 1.—Guinea-pig liver, 3 weeks following injection of normal liver and Freund's adjuvant; extensive granulomatous reaction. H. and E. ×42.

FIG. 2.—Guinea-pig liver, 3 weeks after injection of normal liver and Freund's adjuvant; granuloma with epithelioid cells. H. and E. ×230.

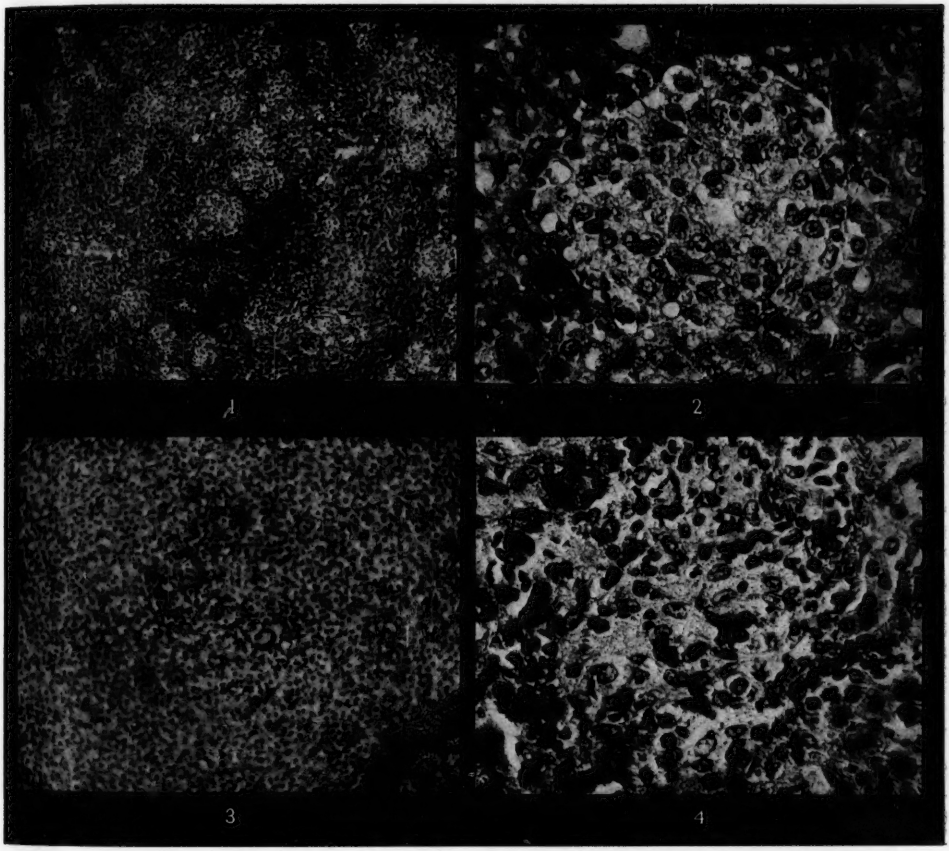
FIG. 3.—Guinea-pig liver, 3 weeks after injection of normal liver and Freund's adjuvant; giant cells. H. and E. ×48.

FIG. 4.—Guinea-pig liver, 3 weeks after injection of normal liver and Freund's adjuvant; granuloma with central necrosis and peripheral infiltration with lymphocytes and histiocytes. H. and E. ×230.

FIG. 5.—Guinea-pig liver, 3 weeks after injection of normal liver and Freund's adjuvant; reticulum network within epithelioid granuloma. Gomori silver stain. ×230.

FIG. 6.—Guinea-pig spleen, 3 weeks after injection of normal liver and Freund's adjuvant; multiple granulomas. H. and E. ×42.

FIG. 7.—Guinea-pig liver, 4 days after injection of normal liver and Freund's adjuvant; acidophilic degeneration of liver cells and focal accumulation of mononuclear cells. H. and E. ×230.



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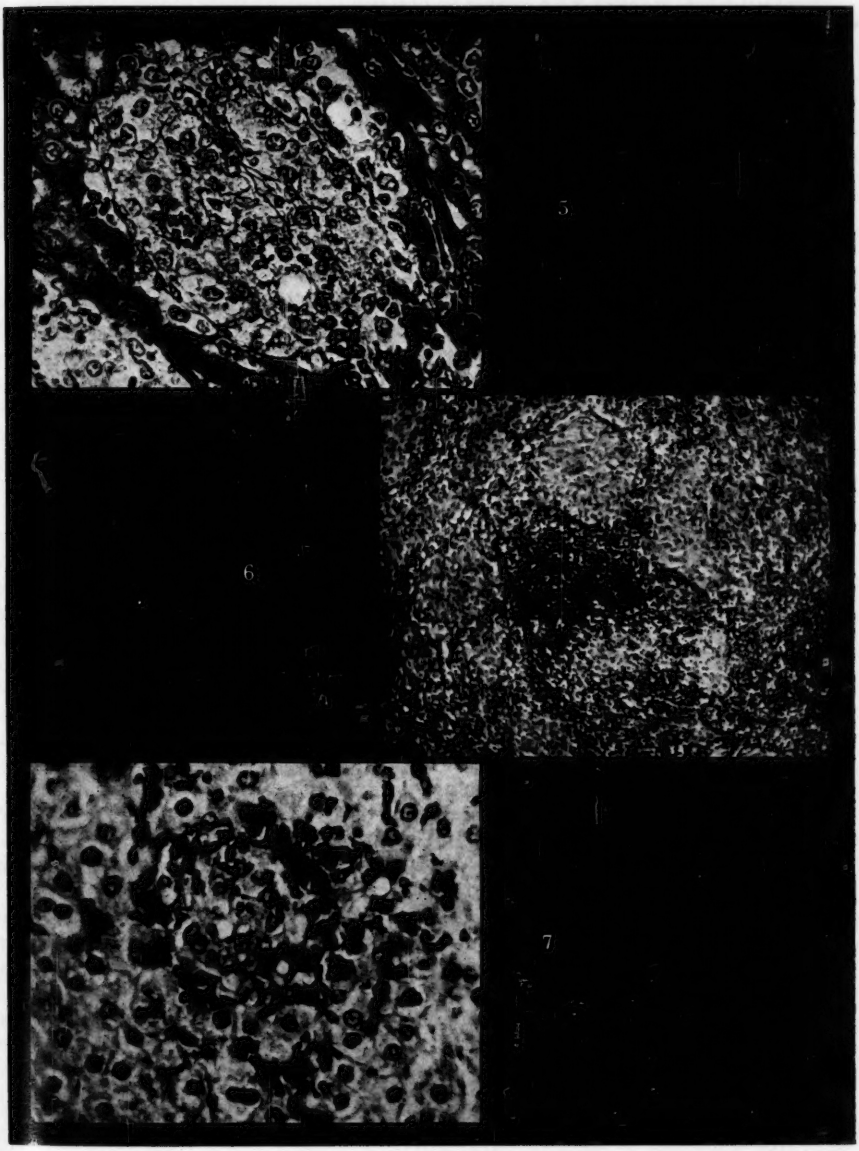

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an early stage and of epithelioid cells at a later stage. This lesion is easily differentiated from the occasional necrotic foci found in controls. The discrete foci of hepatic necrosis and fatty change in animals killed at 4 days and the reticulum stain in animals killed at 3 weeks suggest that liver cell necrosis plays a role in the pathogenesis of the granulomas and may be the initial lesion. Of the 4 possible mechanisms for the pathogenesis of the granulomas, namely : (1) reactivation of a latent infection, (2) embolization of Freund's adjuvant material, (3) hypersensitivity to a constituent of Freund's adjuvant or of the guinea-pig's own tissues and (4) toxic effect on hepatic cells and/or stimulatory effect on the Kupffer cells, the first 2 mechanisms are unlikely because of the negative microbiological stains and cultures and absence of lipid emboli. The presence of focal lesions 4 days after injection speaks against hypersensitivity, at least in the early stages. The results thus support. by elimination, the 4th mechanism and raise the following questions : (1) is the focal accumulation and differentiation of histiocytes secondary to the hepatic necrosis or does it result from an independent stimulation by Freund's adjuvant? and (2) are different components of the adjuvant mixture responsible for the necrotizing and RES stimulatory effects on the liver? The latter question can possibly be answered by separate use of the components of Freund's adjuvant as in the experiments of Laufer *et al.* (1959) and by killing the animals during the first 2 weeks instead of at 5 weeks.

The addition of liver, whether normal or necrotic, to Freund's adjuvant did not qualitatively alter the lesion. The experiments thus fail to confirm the findings of massive necrosis of Behar and Tal (1959). This does not exhaust the experimental approaches to auto-immune liver diseases. Rather, it directs future experiments to the following : (1) Effect of repeated injections ; it has been claimed that numerous injections of homologous kidney in Freund's adjuvant produce a nephritis in rats whereas a single or a few injections were without effect (Heymann, Hachel and Hunter, 1960) and (2) Use of autologous liver since more severe and consistent adrenal lesions in guinea-pigs were obtained following admixture of autologous rather than homologous adrenals to Freund's adjuvant (Steiner, Langer, Schatz and Volpe, 1960).

The numerical increase of hepatic granulomas found by addition of liver to Freund's adjuvant can be interpreted in several ways : (1) an unusual chance distribution ; (2) a hypothetical auto-immune effect and (3) an enhancement of the Freund's adjuvant effect unrelated to the antigenic properties of liver.

These experiments seem to bear on granulomatous liver disease in man. The resemblance of the Freund's adjuvant granulomas to those of sarcoidosis has been pointed out (Chase, 1959). Our experiments add a further similarity, namely the necrosis in the early stages of the lesion (Shay, Berk, Sones, Aegerter, Weston and Adams, 1951). By analogy with the increased frequency of granulomas when hepatic tissue was added to Freund's adjuvant, one may raise the possibility that tissue breakdown products contribute to the pathogenesis of the sarcoid granulomas.

SUMMARY

Guinea-pigs were given a single intramuscular injection of Freund's adjuvant with or without homologous liver and were killed from 4–60 days later. Both groups of guinea-pigs developed focal hepatic lesions which were mainly necrotic at an early stage and formed later epithelioid cell granulomas. The addition of homo-

logous liver to Freund's adjuvant increased the number of granulomas but did not alter the structure of the lesion.

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PROTEIN RELEASE BY TISSUE SLICES (ESPECIALLY RAT LIVER) UNDER VARIOUS METABOLIC CONDITIONS

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Received for publication February 8, 1961

KAUFMANN AND WERTHEIMER (1957) have found that liver slices from 18-hr. fasted rats released more protein into an artificial medium than slices from fed rats (the " fasting protein release phenomenon "). The ability of liver slices to retain a certain fraction of protein was associated with a normal state of carbohydrate metabolism. Release of additional protein was found in states of deficient carbohydrate metabolism. In this article the influence of different physiological and pathological conditions on the release of protein from liver slices from fed and fasted rats was studied.

MATERIALS AND METHODS

Male and female albino rats from the Hebrew University stock, weighing 100–150 g. at 2–3 months of age, were used. Rats (3–4 week-old) weighing 35–40 g. and rats weighing 65–70 g. (6–7 week-old) were used in special cases. Other rodents examined were: male albino mice weighing 25 g.; golden hamsters weighing 80–90 g.; guinea-pigs weighing 250–300 g. and rabbits weighing 2–3 Kg.

The animals were maintained on standard diet. In the basic experiments the rats were starved for 18 hr. during the night before the experiment. In order to obtain varying concentrations of protein, casein was added to the protein-free diet previously described (Kaufmann and Wertheimer, 1957), concomitantly reducing the starch content.

Alloxan diabetes was induced in rats as described previously (Kaufmann and Wertheimer, 1957). Thyrotoxicosis was produced by the subcutaneous injection of 0·4 mg. DL-thyroxine daily for one week (DL-thyroxine, S.A.F. Hoffman-La Roche, Basle, Switzerland). Cortisone-acetate (5 mg. daily) was injected subcutaneously for 2 days before the animals were killed. Carbon tetrachloride (0·8 ml.) was given subcutaneously.

The experiments were carried out as described (Kaufmann and Wertheimer, 1957). The amount of protein released was measured with the Folin-Ciocalteu reagent, which had been calibrated against a standard γ-globulin preparation (Armour and Co., Bovine γ-globulin, Fraction II). The difference in protein release from liver slices of fed and fasted rats may also be calculated on the basis of dry fat-free tissue weights (Itzhaki and Wertheimer, 1957) although this method of calculation was not routinely adopted.

Protein release from liver slices was also determined after perfusion of the isolated liver with oxygenated physiological saline. The results were essentially the same as with the non-perfused preparations although the absolute values of protein released in fed and fasted rats were somewhat lower in slices from perfused liver. Since there was no principal difference, perfusion experiments were not done routinely.

The same chemical procedures used by Kaufmann and Wertheimer (1957), were adopted except that glycogen was determined with the anthrone reagent (Seifter, 1950) after precipitation and washing according to Good, Kramer and Somogyi (1933).

* From a thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the Hebrew University, Jerusalem.

Electrophoresis

After 30 min. equilibration in 4 ml. Krebs-Ringer phosphate medium, the tissue slices were transferred to 4 ml. of fresh medium for a further 3 hr. incubation period.

The medium after incubation was concentrated approximately 50-fold by dialyzing against poly-vinyl-pirollidone (25 per cent). The protein solution was then dialyzed against veronal buffer of ionic strength 0·1, pH 8·6. Electrophoresis was carried out using the same buffer in the Perkin-Elmer model 38A moving boundary apparatus.

RESULTS

Physiological conditions influencing the "fasting protein release phenomenon"

The release of proteins from muscle could potentially serve as an important source of protein during periods of starvation. Diaphragm, extensor digitorum longus (Johnson and Fisher, 1949) and abdominal muscles of rats were investigated for their protein release during periods of fasting up to 6 days. There was no difference in the amount of protein released by muscle of mature rats either fed or fasted. The same may be said for investigations with spleen and kidney. In the latter case, a significant decrease of protein release was found after 5 days of fasting (Table I) instead of the increase which is always found in fasting liver. While Kaufmann and Wertheimer (1957) did not find the "fasting protein release phenomenon" in liver slices of immature rats, this effect was found in muscle tissues of these young rats as may be seen in Table I.

TABLE I.—*Protein Release from Muscle and Kidney Slices*

Tissue examined	mg. protein released/g. wet tissue: Fed	Fasted for 18 hr	*P*
Abdominal muscle (35–40 g. rats)	4·96±0·29 (9)	7·41±0·44 (9)	<0·01
Diaphragm (35–40 g. rats)	8·53±0·34 (14)	11·95±0·25 (14)	<0·001
,, (65–70 g. rats)	11·26±0·89 (7)	14·19±0·75 (7)	<0·03
,, (95–100 g. rats)	11·55±0·67 (7)	13·24±0·26 (7)	<0·05
,, (130–150 g. rats)	11·70±0·55 (13)	12·90±0·75 (14)	>0·2
Kidney (120–150 g. rats, 2–4 days' fast)	10·47±0·33 (20)	10·00±0·39 (32)	>0·3
,, (120–150 g. rats, 5–6 days' fast)	—	8·83±0·32 (18)	<0·01

Numbers in parentheses indicate the number of animals tested.

A significant difference was found in protein release from liver of male and female rats. In 11 experiments the following amounts of protein (mg./g. wet tissue) were released from liver slices of fed rats: 6·04 ± 0·42 in females, 8·47 ± 0·25 in males ($P < 0·01$); from liver slices of fasted rats, 10·92 ± 1·25 in females, 14·86 ± 0·44 in males ($P = 0·02$). This difference could still be demonstrated in tissues removed 5 weeks after castration of either male or female rats. Administration of sex hormones to either normal or castrated animals likewise did not abolish the difference in protein released from livers of male or female rats even after 10 days of administering the hormones.

The "fasting protein release phenomenon" is quantitatively not the same in all rodents. Liver slices from guinea-pigs and rabbits behave much the same as those from rats, while in mice the "fasting protein release phenomenon" can be demonstrated only after intragastric administration of glucose to the fed animals before the experiments. At the other extreme, the golden hamster shows this effect only after a prolonged period of fasting (5–8 days). The difference is probably

related to differences in metabolic rate and to the stability of glycogen reserves in liver of the golden hamster, even after 5 days of fasting, some glycogen could still be found in hamster livers.

Continuing the former experiments (Kaufmann and Wertheimer, 1957), it was shown that galactose and fructose, like glucose, when administered by stomach tube to fasted rats, decreased protein release while pentoses did not. However, the increased protein release in fasted liver slices was not inhibited when glucose was added *in vitro*, while fructose and pyruvate did inhibit the increased protein release. In 8 experiments with liver slices from fasted rats the protein release in a control medium was $19{\cdot}30 \pm 0{\cdot}79$ mg./g. In the presence of 200 mg. per cent fructose, the protein release was $16{\cdot}27 \pm 1{\cdot}16$ mg./g. ($P < 0{\cdot}02$). This fructose effect was also seen in liver slices fron 9 diabetic fed rats (protein release : control $13{\cdot}24 \pm 0{\cdot}42$; with fructose $7{\cdot}90 - 1{\cdot}11$ mg./g., $P < 0{\cdot}01$).

Iodoacetate 5×10^{-4} M, potassium cyanide 2×10^{-3} M and 2,4-dinitrophenol 1×10^{-4} M, and anaerobiosis (100 per cent nitrogen atmosphere) caused a significant increase of protein release from liver slices of both fed and fasted rats. The level of protein in the diet (from 20–70 per cent) influenced only protein release from liver slices of fed rats (Table II), but did not influence protein release from liver slices of rats fasted 1 day after being previously maintained on diets containing various levels of protein.

TABLE II.—*Relationship Between Protein Content of the Diet and Protein Released from Liver Slices mg. Protein/g. Wet Tissue*

	Casein in diet (per cent)		
	20	50	70
Fed rats	$6{\cdot}96 \pm 0{\cdot}48$ (15)	$9{\cdot}04 \pm 0{\cdot}60$ (15) $P < 0{\cdot}02$	$11{\cdot}30 \pm 1{\cdot}77$ (5) $P < 0{\cdot}05$
Fasted rats (18 hr fast) . . .	$19{\cdot}62 \pm 1{\cdot}03$ (17)	$19{\cdot}09 \pm 1{\cdot}08$ (17) $P > 0{\cdot}7$	$22{\cdot}36 \pm 1{\cdot}74$ (5) $P > 0{\cdot}1$

P was calculated for the mean release at the 20 per cent casein diet.
Numbers in parentheses indicate the number of animals tested.

Influence of various pathological conditions on protein release from liver slices

Chronic diabetes.—In addition to former experiments (Kaufmann and Wertheimer, 1957) it has been found that in chronic diabetes of about 3 weeks' duration, when blood sugar levels of fed rats were greater than 500 mg. per cent, the protein release from liver of fed rats was as great as that from fasted rats.

Thyrotoxicosis.—After one week of daily administration of thyroxine, the protein release from liver slices of fed and fasted rats was as high as from liver slices of normal fasted rats. The influence of thyroxine could be seen as early as 12 hr. after the first injection, and after 20 hr. the effect was prominent. At the same time the basic metabolic rate had already risen and liver glycogen stores had decreased. On the other hand, in hypothyroidism, caused by administration of propylthiouracil, the protein release from slices of fasted as well as of fed rats decreased (Kline, 1949).

As may be seen in a typical experiment in Fig. 1, administration of glucose by stomach tube to normal fasted rats caused a decrease in protein release to values

approaching that of fed rats. Similar treatment of fed diabetic rats caused no change in protein release while in thyrotoxic rats, administration of glucose by stomach tube resulted in a drop of protein release during the first 2 hr., followed by a return to the high levels of fasting in the next hour.

"*Refeeding effect*".—Refeeding after a period of fasting caused a marked decrease in the release of protein from liver slices. The amounts released were significantly lower than those from normal untreated fed rats (Kaufmann and Wertheimer, 1957). In alloxan diabetes and thyrotoxicosis this refeeding effect cannot be seen while cortisone had no influence on it (Table III).

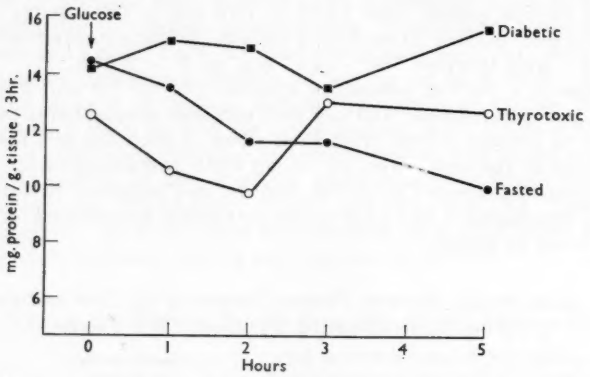

FIG. 1.—Protein release from liver slices of normal fasted rats, diabetic fed rats and thyrotoxic fed rats after glucose administration.

TABLE III.—*Protein Released from Fed and Refed Rat Liver Slices, in Normal and Various Pathological Conditions*

Animals	mg. protein/g. wet tissue	*P*
Normal fasted rats (18 hr. fast)	14·86±0·44 (57)	<0·001
Fed rats		
Normal	8·47±0·25 (61)	..
Diabetic (chronic)	12·87±1·18 (5)	<0·01
Thyrotoxic	12·68±0·51 (7)	<0·001
Cortisone treated	7·75±0·49 (8)	>0·2
Re-fed rats		
Normal	4·29±0·26 (30)	..
Diabetic	11·69±0·87 (3)	<0·01
Thyrotoxic	11·98±1·13 (9)	<0·001
Cortisone treated	3·83±0·45 (8)	>0·3

P was calculated against the normal release in each group.
Numbers in parentheses indicate the number of animals tested.

Liver intoxication.—Three phases of protein release were observed after administration of CCl_4: first, there was an increased protein release in the first hours after intoxication as a direct consequence of liver cell damage. This could have been caused by an increased release of various protein components of the cell. At the

same time binding of new protein might also have been inhibited. Second, there was a marked decrease in release of protein which, according to preliminary experiments (Kaufmann and Wertheimer, 1957), is associated with the regeneration of the liver cells. The third and final phase was characterized by a return to the normal amount of protein release, a sign of the reversibility of the pathological process. It should be noted also that in this model experiment as in liver *in vivo* the return to normal is more rapid with liver slices of fed rats than with those of fasted rats. A typical experiment is given in Fig. 2.

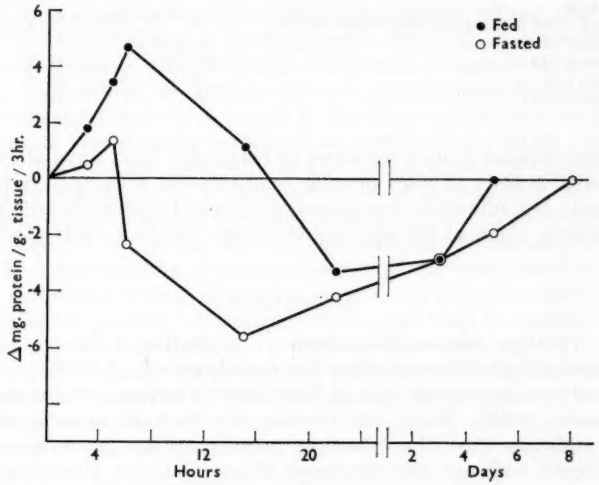

FIG. 2.—Effect of carbon tetrachloride on protein release from liver slices of fed and fasted rats. The values indicate the difference between the amount of protein released (Δ mg./g. wet tissue) in treated and untreated rats, hours or days after injection.

Electrophoretic characterization of the released protein

In a preliminary publication (Gassner-Salgo, Shafrir and Wertheimer, 1957) it was reported that at least 7 components are discernible in the electrophoretic patterns. They have been classified into two groups : 3 components with mobilities up to $-2{\cdot}7 \times 10^{-5}$ cm.^{2} × volt^{-1} × sec.$^{-1}$, and at least 4 components having mobilities between $-3{\cdot}0$ and $-7{\cdot}0 \times 10^{-5}$ cm.^{2} × volt^{-1} × sec.$^{-1}$. These two groups of proteins were referred to as the slow and fast moving components.

The amount of protein in both groups was calculated by multiplying the relative concentration obtained from the electrophoretic patterns by the total amount of protein released.

It was found that the amount of fast-moving proteins released from liver slices of fasted rats was consistently larger than that released from liver slices of fed rats (Table IV). On the other hand, the amount of slow moving proteins released was similar in both conditions. The distribution of the protein components released from thyrotoxic and diabetic tissues of fed rats was similar to that released from tissues of fasted normal rats.

TABLE IV.—*Classification of the Electrophoretic Components of the Released Protein as Indicated by Their Mobilities*

Specification of animals	Number of rats	Protein mg./100 ml. Group of fast components	Protein mg./100 ml. Group of slow components
Fed rats	10	5·7	35·9
Fasted rats (18 hr. fast)	9	24·6	29·2
Rats injected daily for one week with 0·4 mg. DL-thyroxine	12	28·2	29·8
Rats injected with 0·4 mg. DL-thyroxine 20 hr. before being killed	9	23·4	37·1
Alloxan diabetic rats	12	16·9	32·8
30–40 g. rats, fed	5	10·6	15·9
30–40 g. rats, fasted	5	14·6	19·4

The protein released from liver slices of rats refed for 2 days after 2–5 days of fasting consisted mainly of slow-moving components with only a trace of fast-moving protein. No difference was found in the electrophoretic pattern of protein released from liver slices of fed and fasted young (35–40 g.) rats (Table IV).

DISCUSSION

That the " protein release phenomenon " in fasting is specific for liver slices has been re-established. One exception was found, namely, it could be demonstrated in muscle of very young rats but not in liver slices of animals at this age (Kaufmann and Wertheimer, 1957). Since the reverse was seen in mature animals, these experiments indicate that the " fasting protein release phenomenon " in liver develops gradually with age, as it decreases in muscle tissue, it gradually appears in liver. It seems that protein in structurally immature muscle may serve as a source of special reserve protein.

From liver slices of acute and chronic severe diabetic fed rats the protein release was far greater than from liver slices of normal fed rats. Administration of glucose and fructose to diabetic rats *in vivo* could not cancel this effect, but addition of fructose *in vitro* diminished significantly the protein release of liver slices even from diabetic fed rats. This is understandable since *in vivo*, fructose is rapidly converted to glucose (Banerjee and Eswaramangalath, 1957) while *in vitro*, fructose is, to a great extent, oxidized by liver slices directly without prior conversion to glucose and without the necessity of insulin. This disturbance is still more pronounced in refed rats when examined in normal and diabetic state. It is indeed the same as in carbohydrate deficiency (Kaufmann and Wertheimer, 1957), only in the latter case glucose administration decreased the release while in diabetes it did so only when administered together with insulin.

It may be presumed that in thyrotoxicosis, as a consequence of the increased metabolic rate, a higher rate of carbohydrate metabolism is necessary in order to restore the normal protein release from liver slices of fed rats.

The fasting, diabetes, thyrotoxicosis and refeeding experiments demonstrate again the importance of carbohydrate metabolism in protein binding and release in liver cell (see also Kaufmann and Wertheimer, 1957). It seems justifiable to hypothesize that the inhibition of protein release in hunger by glucose might be

due to an increase in synthesis of microsomal-bound protein in the light of the finding of Keller and Zamecnik (1955).

In the presence of DNP, cyanide etc., liver slices have shown a greater protein release. This probably represents an inhibition of protein synthesis and binding. That new protein synthesis is a necessary requirement for protein binding was also suggested by experiments with ethionine feeding. When 0·4 per cent ethionine was added to the regular diet for 2 weeks, a rapid decrease in body weight resulted. Protein release from liver slices was measured and in 14 experiments with fed rats, it was $10{\cdot}22 \pm 0{\cdot}56$ mg./g. compared to $6{\cdot}27 \pm 0{\cdot}51$ in the control rats ($P < 0{\cdot}001$); in 8 experiments with fasted rats, protein release was $10{\cdot}45 \pm 0{\cdot}74$ compared to $11{\cdot}84 \pm 0{\cdot}37$ mg./g. in the control rats ($P > 0{\cdot}1$). Methionine given in the diet concomitantly with ethionine completely reversed the latter's effect.

If there is no synthesis of protein without carbohydrate metabolism and only the newly synthesized protein is bound in the liver cell, it may be said that this structural binding also requires carbohydrate metabolism. From our experiments it is difficult to tell whether protein release as such is an active process.

Electrophoretic analysis of the material released showed that the protein increment from liver slices of fasted rats appeared in the electrophoretic pattern as a quantitative increase of the fast-moving components. It was also found that the increase of these fast-moving protein components was correlated with conditions in which an additional release of protein occurs, *e.g.* thyrotoxicosis and diabetes. Therefore, increased protein release from liver slices may be caused by an impairment of carbohydrate metabolism or a carbohydrate shortage. The role of these fast-moving components as reserved protein in the liver is being further investigated. The electrophoretic patterns of protein released from livers of fed and fasted very young rats were identical. This is in accordance with the finding that the "fasting protein release phenomenon" is absent in very young rats.

SUMMARY

The greater protein release from liver slices of 18 hr. fasted rats as compared to fed rats (the "fasting protein release phenomenon") has previously been found to be specific for liver tissue of mature animals. In very young rats, the "fasting protein release phenomenon" could be demonstrated in muscle tissue but not in liver. In diabetes of short duration and in chronic severe diabetes, the protein release of liver slices of fed animals was increased to a level which cancelled the "fasting protein release phenomenon". Glucose administration without insulin did not neutralize the effect of diabetes. Thyrotoxicosis increased protein release of liver slices in fed animals. Glucose administration decreased this protein release to the normal level but only for a short period. In hypothyroidism the protein release from liver slices of fed and fasted rats was decreased.

Refeeding after prolonged fasting caused a decrease in protein release compared to liver slices of normal fed rats. In diabetic and thyrotoxic rats after refeeding, the protein release was as great as in liver slices from fasted rats. Liver damage due to CCl_4 injection resulted in an initial increase in protein release from liver slices followed by a marked reduction which was fully reversed after several days. Ethionine administration cancelled the "fasting protein release phenomenon" while methionine reinstated it.

The additional protein released on fasting was associated with a rise of the electrophoretically fast-moving components. These were also increased in liver incubates of fed diabetic and thyrotoxic rats. Liver incubates of normal refed rats, showed only a trace of fast-moving components.

The authors wish to thank Mrs. Aya Brudner-Leder, Mr. M. Tal and Dr. Elaine R. Berman for their assistance.

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A STUDY ON THE RESISTANCE OF THE ADRENAL CORTEX TO TUBERCULOUS INFECTION

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Received for publication February 16, 1961

In previous experimental work on the localization of tuberculous infection (Blacklock and Williams, 1957) it was noted *inter alia* that when tubercle bacilli were inoculated directly into the arterial side of the circulation that the adrenals were rarely, or only slightly, involved. Of 110 rabbits so inoculated with approximately 5×10^5 human tubercle bacilli (H37Rv) and killed 22–105 days later, only 22 showed slight lesions in the adrenals on histological examination, even where there were extensive tuberculous lesions in other organs. The adrenal lesions consisted of sharply defined small foci of endothelioid cells with occasionally a giant cell but no necrosis or caseation (Fig. 1). The character of these lesions suggested that the adrenals might be more resistant to tuberculous infection than other organs. This experimental finding was in keeping with that observed by Blacklock (1932) in studies on tuberculosis in the human subject where, though there was extensive organ involvement, the adrenals were largely spared. On the other hand, in cases of Addison's disease where there is complete destruction of the adrenals by fibrocaseous tuberculosis it is unusual to find any other tuberculous lesions in the body, a fact commented on by Addison (1849) in his original description. Sanford and Favour (1956) found that of 125 patients with Addison's disease 94 (75·2 per cent) had no clinical history of tuberculosis. Sloper (1955) reported that of 22 cases who had died of fibro-caseous tuberculosis of the adrenals the presence of active tuberculosis elsewhere in the body was exceptional and that extra-adrenal tuberculous lesions were small and quiescent. No satisfactory explanation of this comparative immunity of the adrenals to tuberculosis has ever been given.

In an attempt to solve this problem, it was decided to study the effect of direct inoculation of a suspension of human or bovine tubercle bacilli directly into the adrenals of rabbits and guinea-pigs.

MATERIALS AND METHODS

A fully virulent strain of human tubercle bacilli (H37Rv) and a strain of bovine tubercle bacilli recently isolated from an ox were used in all these experiments. Suspensions were prepared from 10–14-day-old cultures in Dubos medium, and diluted so that 0·02 ml. contained approximately 5×10^2 bacilli which was used for inoculation. In order to avoid contamination of the wound the needle used for drawing the suspension into the syringe was discarded and a sterile one subsituted. The animals were anaesthetized with nembutal and ether and the adrenal gland on one or both sides exposed through an incision in the flank. The suspension of tubercle bacilli was injected directly into the exposed gland, taking care that no spillage occurred and thereafter the wound was sutured in layers.

RESULTS

Rabbits

The left adrenal of 5 rabbits was inoculated with the *human* strain and 14 days later the right adrenal with the same amount. The animals were killed at intervals of 75–313 days after the second inoculation. In those killed in the earlier period, some tubercles were found along the needle track in the cortex and in the medulla, whereas 300 or more days after inoculation the medulla of the gland was destroyed by caseation in which calcification had taken place (Fig. 3). The lungs, liver, spleen and kidneys of all these animals showed some chronic tuberculous lesions.

Three rabbits were inoculated with the same dose of the *bovine* strain into the left adrenal. These were killed 46–117 days after inoculation. The adrenal in all showed an acute tuberculous lesion localized to the needle track through the cortex, with destruction of part or the whole medulla by caseation (Fig. 4). In addition there was some caseous change in the tissues surrounding the adrenal. The lungs, liver, spleen and kidneys were extensively affected by acute caseating tuberculosis.

In 3 other animals, the same dose was used to inoculate both adrenals, the right being inoculated 14 days after the left. These animals were killed 17–50 days after the second inoculation. The lesions in the adrenals were similar to those in the former group except in the animal killed at 17 days, where only small tubercles (Fig. 5) were found along the needle track through the cortex and in the medulla. These were composed of endothelioid cells and no caseous change was present. In all the animals, all the other organs showed acute tuberculous lesions.

Electrolyte changes

In the rabbits an attempt was made to study the effect on the blood sodium and potassium levels of the inoculation into the adrenals. The blood sodium and potassium was ascertained in a series of 8 normal rabbits, kept under the same conditions and receiving the same food as those under experiment. The normal sodium range was 134–148 m.eq./litre with a mean of 140 and the potassium 5·0–7·6 with a mean of 5·8 m.eq./litre.

TABLE I.—*Rabbit* 68/57—*Killed* 313 *days After Inoculation of Both Adrenals with Human Strain*

	Na milli-equivalents/ litre	K milli-equivalents/ litre
Before inoculation	140	5·55
10 days after inoculation	142	5·4
24 „ „ „	140	5·5
38 „ „ „	140	6·8
54 „ „ „	142	5·4

Other animals inoculated with the human strain gave similar results.

Tables I and II show the changes in the electrolytes in rabbits inoculated with tubercle bacilli into both adrenals. In animals inoculated with the human strain the sodium and potassium levels fell within normal limits, whereas in those inoculated with the bovine all showed a rise in the potassium at the later periods

TABLE II.—*Rabbit* 75/57—*Killed* 50 *Days After Inoculation of Both Adrenals with Bovine Strain*

	Na milli-equivalents/ litre	K milli-equivalents/ litre
Before inoculation	144	6·3
15 days after inoculation	160	6·7
24 „ „ „	134	5·3
31 „ „ „	138	6·9
37 „ „ „	164	7·4
47 „ „ „	162	6·0
49 „ „ „	144	17·0 (animal ill)

Other animals inoculated with the bovine strain gave similar results.

when the animals appeared ill and weak. This late rise is probably the result of a general toxic effect on account of the widespread tuberculous lesions, particularly in the kidneys, rather than a pure adrenal effect.

Guinea-pigs

The left adrenal of a series of 6 guinea-pigs was injected with the *human* strain, the animals being killed 51–65 days later (average 60 days). In all of these a tuberculous lesion was noted along the needle track in the adrenal cortex, with in some an occasional non-caseous miliary tubercle, consisting of endothelioid cells in the cortical zone. Though tubercle bacilli were present, the lesions in the cortical zone were peculiarly localized. In 4 of the animals the periadrenal tissue was caseous. Numerous tuberculous lesions were found in the lungs, spleen and liver of all 6 animals.

The left adrenal of a series of 6 guinea-pigs was similarly inoculated with the *bovine* strain and these animals died on an average 36 days later. In the cortex the needle track showed a localized tuberculous lesion with an occasional, sharply defined, miliary tubercle elsewhere in the cortical zone. In all the animals the medulla was partially or completely destroyed by caseating tuberculosis which involved to a limited extent the inner zone of the cortex (Fig. 2). In some, caseation was present in the periadrenal tissues but this was sharply demarcated from the adjacent healthy cortex. In all these animals the spleen, liver and lungs showed extensive acute tuberculosis.

It appeared from the above results that though the adrenal medulla could be destroyed by the tuberculous infection, yet the cortex was more resistant. It was, therefore, decided to investigate the matter further in guinea-pigs in the following ways :

Injection of ground fresh adrenal into muscle.—It has been shown (Blacklock and Williams, 1957) that tubercle bacilli, following intra-cardiac inoculation, will localize at the site of injury or of injection of such substances as penicillin. If the adrenals were even partially resistant to infection with tubercle bacilli, then it might be due to some substance in the gland which either inhibited the multiplication of the bacilli or influenced the cellular response and the subsequent lesion. Accordingly the adrenals were excised aseptically from a normal guinea-pig, ground in 0·85 per cent saline to form a suspension that could be injected easily through a needle. Within 10 min. of excision this suspension was inoculated

into the thigh muscles of a guinea-pig and immediately thereafter 0·25 ml. of a suspension containing approximately $1{\cdot}25 \times 10^{11}$ human tubercle bacilli was inoculated directly into the left ventricle exposed by thoracotomy. All the 4 guinea-pigs so treated were killed at 58 days, and showed localization of the infection at the site of injection in the muscles of the ground adrenals, with a marked reaction on the part of mononuclear leucocytes (Fig. 6) in addition to tuberculosis in the organs.

Implantation of fresh adrenal into muscles.—The adrenals were excised under aseptic precautions from normal guinea-pigs as before and a whole gland was implanted into the thigh muscles of each of 4 guinea-pigs. Two days later approximately $1{\cdot}25 \times 10^5$ human tubercle bacilli were inoculated into the left ventricle after a thoracotomy. The animals were killed 4–5 weeks later and in all tubercle bacilli were found around the necrotic implant in the granulation tissue in which there were numerous tubercle follicles. The same result was obtained in other guinea-pigs with boiled or autoclaved adrenals. It is thus probable that in the emulsified adrenal or in the whole excised fresh adrenal, the substance which inhibits the lesion in the normal gland is not present in sufficient amounts over a period to prevent localization of the infection or prevent a reaction.

Implantation of cortisone tablets into muscles.—Tablets of cortisone acetate (25 mg.) were implanted into the thigh muscles of 3 guinea-pigs and lactose tablets of the same size into similar muscles of 3 other guinea-pigs. Immediately after the implantation $1{\cdot}25 \times 10^5$ human tubercle bacilli were injected into the left ventricle exposed by thoracotomy. In all the animals tubercle bacilli were found around the implants though in the case of the cortisone implants the cellular reaction was composed of large mononuclears and endothelioid cells, whereas in the case of the lactose in addition to these cells many polymorphs were also present.

In view of the difference in the cellular response, this experiment was repeated but into each of 8 guinea-pigs a cortisone tablet was implanted into the muscles of the left thigh and a lactose tablet into those of the right thigh. On the day of implantation human tubercle bacilli, as in the previous experiments, were inoculated into the left ventricle. The animals were killed at intervals from 2–6 weeks after inoculation. In all tubercle bacilli localized at the site of implants and the

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EXPLANATION OF PLATES

FIG. 1.—Rabbit. Miliary lesions in adrenals 71 days after inoculation of human tubercle bacilli into left ventricle. ×63.

FIG. 2.—Guinea-pig. Direct inoculation of bovine bacilli into adrenals: death 39 days later. Adrenal medulla showing localized caseation. ×2.

FIG. 3.—Rabbit. Direct inoculation of human bacilli into adrenals: killed 313 days later. Adrenal medulla showing caseation and calcification. The fibrosed needle track extends from surface inwards. ×43.

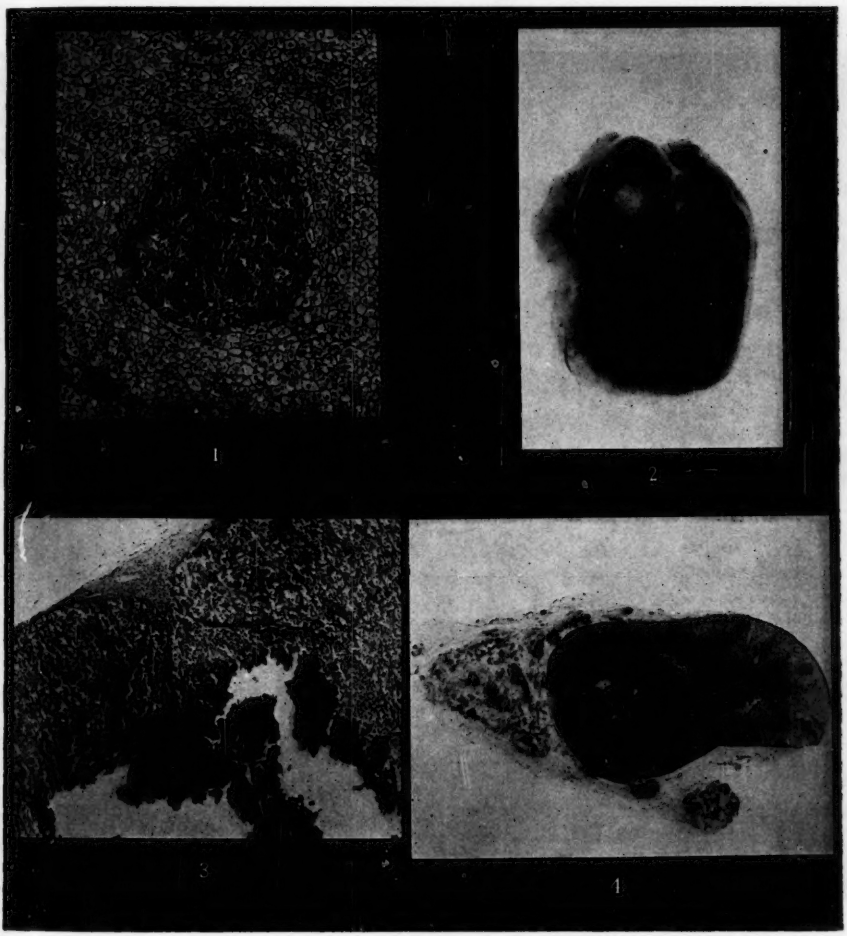
FIG. 4.—Rabbit. Direct inoculation of bovine bacilli into left adrenal: killed 50 days later. Caseating tuberculosis around needle track: three small tubercles in cortex: peri-adrenal caseation. ×5.

FIG. 5.—Rabbit. Small tubercles along needle track in cortex after inoculation of bovine bacilli: killed 17 days later. ×63.

FIG. 6.—Guinea-pig. Reaction at site of injection of fresh ground adrenal into muscle: human tubercle bacilli inoculated into left ventricle: killed 58 days later. ×63.

FIG. 7.—Guinea-pig. Mononuclear reaction around cortisone tablet inserted into muscles: human tubercle bacilli inoculated into left ventricle: killed 2 weeks later. ×230.

FIG. 8.—Guinea-pig. Polymorphonuclear reaction around lactose tablet inserted into muscles: human tubercle bacilli inoculated into left ventricle: killed 2 weeks later. ×230.



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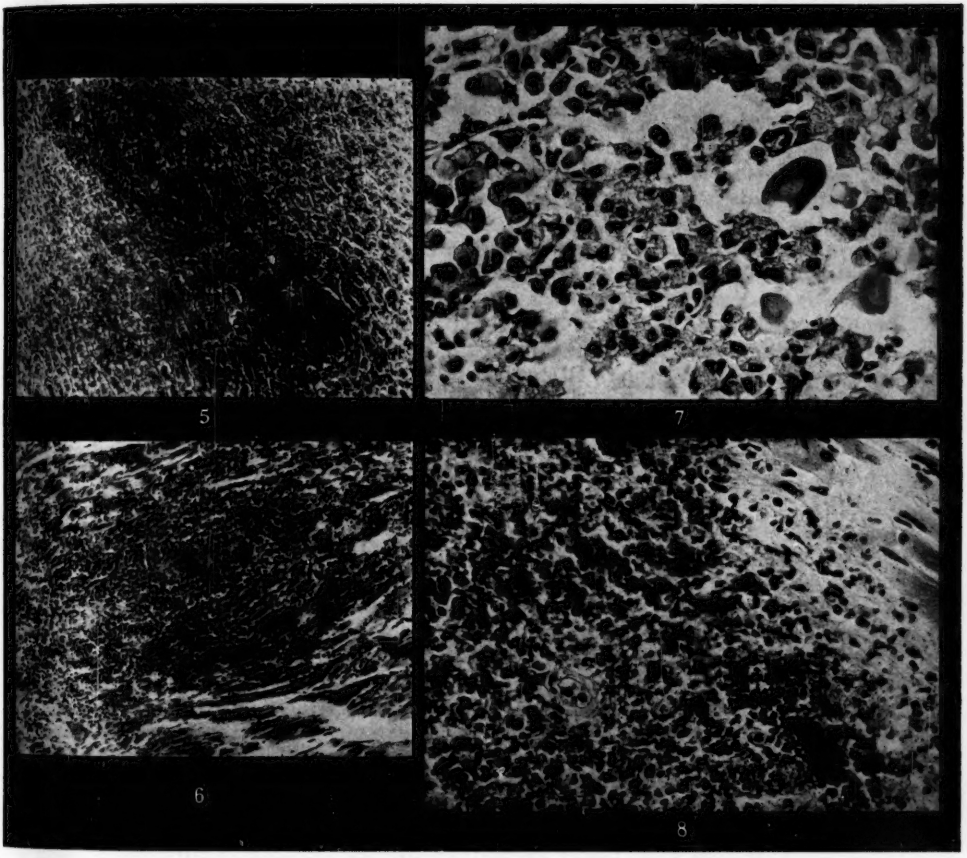

Blacklock and Rees.

number of bacilli as judged in sections stained by Ziehl-Neelsen did not materially differ around the cortisone implant as compared with the lactose. It was obvious, however, that 2–3 weeks after implantation the cellular reaction around the cortisone was less intense and mainly composed of mononuclear leucocytes than around the lactose where the cellular exudate was mainly of polymorphonuclear leucocytes (Figs. 7 and 8). After 4–6 weeks the reaction around the lactose was more intense and diffuse and showed necrosis and caseation, whereas that around the cortisone remained much as observed at the second week.

DISCUSSION

The slow evolution of the lesions produced experimentally in rabbits and in guinea-pigs in the cortex of the adrenal either by injecting tubercle bacilli into the arterial blood stream or by the direct inoculation of these organisms into the adrenals was remarkable as compared with what occurs in other tissues as a result of similar experimental infection. According to Ustvedt (1949) who studied the histories of a series of cases of Addison's disease there is a long interval between the first clinical manifestation of tuberculous infection and the onset of the disease in the adrenals, this interval being more than 15 yr. in 32 of 58 cases. He concluded that the evolution of tuberculosis in the adrenal took a long time and this period of clinical latency was longer in the adrenals than in any other organ. Our experimental results support this view, as in the guinea-pigs which are very susceptible to tuberculous infection the cortex showed little involvement at the site of direct inoculation 65 days after infection with a virulent human strain (H37Rv) and 36 days after an infection with a bovine strain. Even more striking were the results in rabbits in which direct inoculation of the adrenals with a human strain (H37Rv) resulted in a tuberculous lesion limited to the needle track 313 days later, and 117 days later with a bovine strain. This bovine strain was fully virulent for the rabbit, killing in 42 days with generalized tuberculosis after intravenous inoculation of 0·01 mg. of moist culture. Minimal lesions also resulted in the adrenal cortex 22–105 days after human or bovine tubercle bacilli were inoculated directly into the arterial side of the circulation : these lesions consisting of foci of endothelioid cells.

The very localized lesions produced in the adrenal cortex in these experiments is due to one of two causes or to a combination of both. First the normal reactive mechanism to infection is altered by the hormones present in the cortex. Thus no damage to the capillary blood vessels was observed. There was no exudate, little necrosis and no polymorphonuclears in the lesions, the cells of which consisted of mononuclears and endothelioid cells. Second, the cortical hormones would depress any allergic hypersensitivity around the lesions and thus limit or even prevent exudate and spread, a very localized lesion resulting. It is possible that as time passes and provided the tubercle bacilli are still viable that this depression of hypersensititivity may become less and thus the lesion may then increase in size and caseate. On the other hand when the medulla is involved none of these factors is present and the lesion produced eventually involves and destroys the whole of that structure. Ebert (1952) made very similar observations using the ear chamber technique in rabbits infected with tuberculosis but receiving cortisone.

Under the hormonal conditions prevailing in the adrenal cortex, experimental tuberculous lesions would either tend to retrogress, as they actually did in rabbits

—where calcification took place—or progress very slowly, thus supporting the observations of Ustvedt quoted above in the human subject.

It was hoped that the changes in the electrolyte balance would be a guide to the progress of the lesions in the adrenals during the life of the animals, but owing to the very limited size of the lesions, this did not prove to be the case.

In experiments where fresh whole or ground adrenal was implanted in the tissues and a tuberculous bacteraemia induced thereafter, the conditions did not altogether resemble those in the adrenal cortex where the concentration of hormones is more or less constant. As the hormones in the implanted tissues decreased, tubercle bacilli localized in the area, causing quite distinct lesions.

The same was found to be true when cortisone tablets were introduced into the tissues, though here the resulting lesions were less than those around the lactose tablets which had been used as controls. This, however, was not a clear-cut experiment as, in addition to the trauma caused by the insertion of the tablets into the muscle, the vehicle used to make up the bulk of these would act as a foreign body unless soluble. In both cases doubly refractile bodies were found at the site of implantation, suggesting that the vehicle was not quickly soluble and, therefore provoked a foreign body reaction in which tubercle bacilli, if present in the blood stream, are liable to localize (Blacklock and Williams, 1957).

SUMMARY

Lesions produced in the adrenals after infection of the arterial blood or direct inoculation with virulent human or bovine tubercle bacilli were minimal in the cortical zone and more extensive in the medulla. No change in the electrolyte balance resulted. Experiments to explain the slow evolution of the cortical lesions indicated that in the living adrenal a hormonal factor is responsible. The long interval between infection and the manifestations of Addison's disease are probably due to this factor.

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A CINEMATOGRAPHIC STUDY OF *TOXOPLASMA GONDII* IN CELL CULTURES*

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Received for publication February 23, 1961

In a previous report propagation of *Toxoplasma gondii* in cell cultures was described (Sourander, Lycke and Lund, 1960). The observations were made by means of phase contrast microscopy. It was shown that the life cycle may be completed within 48 hr. Therefore it seemed advantageous to employ time lapse technique for a detailed study of the penetration of the parasites into the cell, their multiplication and the subsequent rupture of the cell with liberation of the newly formed parasites.

MATERIAL AND METHODS

Culture technique.—The cells were cultivated in chambers as described by Gey. The bottom of the chamber was an ordinary microscope slide. A ring of stainless steel, 0·75 mm. thick and with an inner diameter of 35 mm. was glued to this by means of bees wax. About 0·5 ml. of cell suspension was placed inside the ring. Thus a quarter of the chamber space was taken up by the fluid. The top of the chamber consisted of a cover slip, sealed with vaseline. This type of chamber was used with low power magnification. For use with higher magnification both sides of the chambers consisted of cover slips. For use with oil immersion microscopy it was necessary to omit the metal ring so that the chamber consisted only of two cover slips sealed with vaseline. In this type of culture the cells could be maintained in good condition only 48 hr.

Biological material.—Cancer cells of the HeLa strain and trypsinized human embryo kidney cells were used throughout the experiments. The cell suspension contained about 50,000 cells per ml. The cells were cultivated in a liquid medium containing 15 per cent dye test negative human or calf serum in Hanks' solution with 0·5 per cent lactalbumin hydrolysate at 37°. The medium contained 50 units of penicillin and 50 μg. of streptomycin per ml. As a rule the cultures were inoculated 1–5 days after establishment. The source of parasites was freshly harvested, peritoneal exudate from mice infected with the RH strain of *Toxoplasma gondii*. The cell culture fluid was replaced by a 1 : 5 dilution of exudate in medium. After 1–2 hr. the culture was rinsed and new medium added in order to avoid toxic effects of the exudate.

Cinematographic technique.—For the film work a Zeiss Mikrokinokamera was used. The source of light was a 12 volt, 6 amp microscope lamp. The magnification of the phase objective used for general observations was 25 times. The growth of the parasite clones was followed using a 40 × objective whereas details of the parasites were studied with oil immersion, 100 × objective. The corresponding magnifications on the film were 75, 120 and 200 times. The action was speeded up with a factor varying from 1–250 times. A Kodak Plus X-negative, 16 mm. film was used. From this positive prints were made for illustrations.

RESULTS

As previously observed (Sourander, Lycke and Lund, 1960) the toxoplasma parasites are seen in the cytoplasm within 15 min. after the inoculation of the

* This study was supported by a grant from Statens Medicinska Forskningsråd.

cultures and sometimes even sooner. Mostly these parasites are seen in a juxtanuclear position. At this stage the parasite shows a well defined nucleus with a distinct, single nucleolus. In the cytoplasm of the parasite dark appearing granules of varying size are seen (Fig. 1*a* and *b*). These granules are rapidly moving. In most cases the pointed end shows one or two elongated rod-shaped inclusions and at the blunt end a more rounded one is found, close to the parasite's nucleus. This inclusion could be found moving together with the rotating nucleus as if attached to the nucleus. Sometimes minute, light refracting spots resembling vacuoles are observed in the cytoplasm of the parasite (Fig. 2).

The parasite increases in size and a vacuole is formed around it. During the growth phase the nucleolus of the parasite disappears, the nucleus increases in size and the nuclear boundary appears less distinct. Later on, nuclear division occurs giving rise to 2 nuclei with well defined nucleoli. The division of the nuclei is completed in 15 min. In 30–45 minutes the division of the whole parasite is accomplished (Fig. 3*a*–3*d*). The cleavage seems to follow along an oblique axis between the 2 nuclei. This explains the asymmetry in configuration of the parasite pair when seen from different angles. Subsequently, the parasites turn so that the blunt ends are facing each other and a coffee bean like structure is formed (Fig. 4*a*–4*k*). The first division of the parasite is completed in 3–4 hr. after penetration of the cell. Later the generation time seldom exceeds 1 hr.

Repeated divisions of the parasites follow the same pattern. As the parasites increase in number the vacuole increases in size and occasionally may occupy the whole cytoplasm dislocating the cell nucleus. The individual parasites within the clone seem to undergo synchronous divisions but sometimes it may take as much as 2 min. before all parasites of the same generation are divided. The parasites regularly form a flower-like pattern. The characteristic structure of the clone disappears during multiplication of the parasites but is often reorganized by the newly formed ones. The clone is surrounded by a distinct membrane-like structure (Fig. 5). Many cells contain more than one clone as the cells frequently are invaded by several parasites and each of these give rise to a separate clone.

After a number of parasite divisions a single cell harbours a huge number of parasites. When a critical stage is reached rupture of the cell follows immediately after a division of the parasites (Fig. 6*a*–6*c*). This event, usually occurring 24–48 hr. after inoculation, is not dependent on a fixed number of parasites but more on the localization of the clone in the cytoplasm. Burst of a cell containing only a small clone has been observed when the clone was localized in a narrow space between the nucleus and the cell membrane. The liberated parasites are of uniform size and actively moving. A fast, initial speed amounting to 3 mm. per sec., *i.e.* 400 times the parasite length per sec. was recorded, However, in a few minutes this activity is slowed down. Principally, the movements seem to follow in the direction of the parasite's long axis. A single cell yield may contain about 200 parasites. Within a few seconds the adjoining cells are massively invaded, but some parasites are found attached to cell surfaces, to cellular debris or clumped together. Shortly after the penetration, growth and multiplication of parasites are initiated. Now even the first division occurs within one hour. Otherwise the multiplication proceeds in the same manner as described above.

The nucleus of the disrupted cell seems to be structurally well preserved. In no cases parasites were observed inside the nucleus but often the shape of the nucleus might be deformed. Mitosis of infected cells were frequently observed.

When a cell containing a single clone divided one of the daughter cells was found non-infected while the clone reappeared unchanged in the other one. However, occasionally after the division of the nucleus the division of the cytoplasm was interrupted. The cleavage was not completed in that part which contained a large clone.

DISCUSSION

Already Nicolle and Manceaux (1909) reported binary fission to be the mode of multiplication of the *Toxoplasma gondii*. They found paired parasites in the cytoplasm before the cell ruptured. Splendore (1908, 1909) described multiplication of the parasites as schizogony. However Laveran (1915) stated that binary fission was the only mode of multiplication. Cross (1947) described the binary fission as longitudinal and so did Schmidt-Hoensdorf and Holz (1953) using cinematographic technique. However, also transverse division of the parasites was reported in the electron microscopic studies performed by Bringmann and Holz in the same year. Westphal (1954) claimed that the rosettelike structure arises as a result of a single multiple division.

The time lapse technique, as used in the present study, enabled detailed observations of the parasite division. In our observations the parasites divided regularly by binary fission. As mentioned above, the cleavage of the parasite seemed to follow along an oblique axis. Thus, the cleavage, if observed from a certain angle, might appear as a transverse fission. This phenomenon may be the explanation of the observations by Bringmann and Holz in their electron microscopic study. Sometimes one even got the illusory impression that a multiple division was occurring. However, careful study of the films revealed that this impression was due to the twisting of the parasites during the division and, actually, when the division was completed only 2 parasites had been formed. During the almost synchronized divisions of the parasites in the clone the characteristic flower-like pattern of the clone disappeared. Again, it momentarily appeared as if a great number of smaller parasites were present. At this intermediate stage the picture resembled the ones described and interpreted by Westphal as stages of multiple divisions during the acute phase of infection.

Vacuoles surrounding the parasites were described already by Guimarães and Meyer (1942). Mühlpfordt (1952) pointed out that these vacuoles could probably not be artefacts as they were also observed in living cells. They were not seen in uninfected cells and their size was depending on the number of parasites. Vacuoles were also described in the electron microscope study of Gustafson, Agar and Cramer (1954). In phase contrast microscopy a definite dark boundary was seen around the vacuoles (Sourander, Lycke and Lund, 1960). The same boundary appeared darkly stained in fixed preparations.

It is tempting to speculate whether the boundary is actually a membrane. In high power magnification it is seen to follow closely the shape of the clone (Fig. 5). In fact, one gets the impression that the regularity in the structure of the clones is more a result of the presence of a membrane rather than due to internal organization. The flower-like pattern of the clone disappears during multiplication when movements of the parasites are observed. The pattern of the clone is often reorganized when multiplication is completed. Sometimes, however, the symmetrical arrangement of the parasites is not accomplished but they are anyway packed closely as if held together by a membrane.

According to Cross the intracellular parasites increase in size and undergo recurrent and alternating periods of growth and binary fission until a maximum in growth is reached beyond which the mass of the parasites is not further increased. The parasites in what he interprets as terminal colonies are reported to be smaller than those in earlier stages of invasion. Mühlpfordt reported " two different sizes of toxoplasmas, large ones situated in plasma vacuoles and smaller ones in the cellular plasma ".

In agreement with the observations by Cross we have found that the single parasite before division increases in size. However, there seemed to be no decrease in size as a consequence of repeated divisions. Parasites of first, second, third and fourth generations were of the same size at corresponding stages of growth. It is of course difficult to determine the size of the parasites in a crowded clone. But it was observed that parasites liberated when the cells ruptured, were of a size equal to that of the parasites first invading the cells (Fig. 6).

The term " terminal colony " has been used to designate the final stage of cell parasitization. If used to define the status of the parasite invaded cell immediately before rupture of the cell, the term must be considered inadequate as according to our observations the rupture of the cell seems to be closely depending on multiplication of intracellular parasites. It is our impression that the cell ruptures at the stage when the elastic capacity of the cell membrane is exhausted due to parasite multiplication.

All earlier authors seem to agree that the newly formed parasites are liberated after rupture of the host cell. However Schmidt-Hoensdorf and Holz studying toxoplasma infected macrophages in cover slip cultures claimed that the liberation of the toxoplasma is a result of active screw movements of the single parasites.

EXPLANATION OF PLATES

FIG. 1*a*–1*c*.—Toxoplasma parasites in human embryonic kidney cells. Fig. 1*a*. In the cytoplasm of the cell close to the nuclear membrane 3 pairs of parasites are seen. ×4090. Fig. 1*b*. shows a cell infected with several parasites. ×1230. Fig. 1*c* shows the well-defined nucleoli of the parasites as well as dark appearing granules in the cytoplasm of the parasites.

FIG. 2.—Toxoplasma parasites in a HeLa cell. Note the minute light refracting spots in the cytoplasm of the parasites and dark inclusions. ×1230.

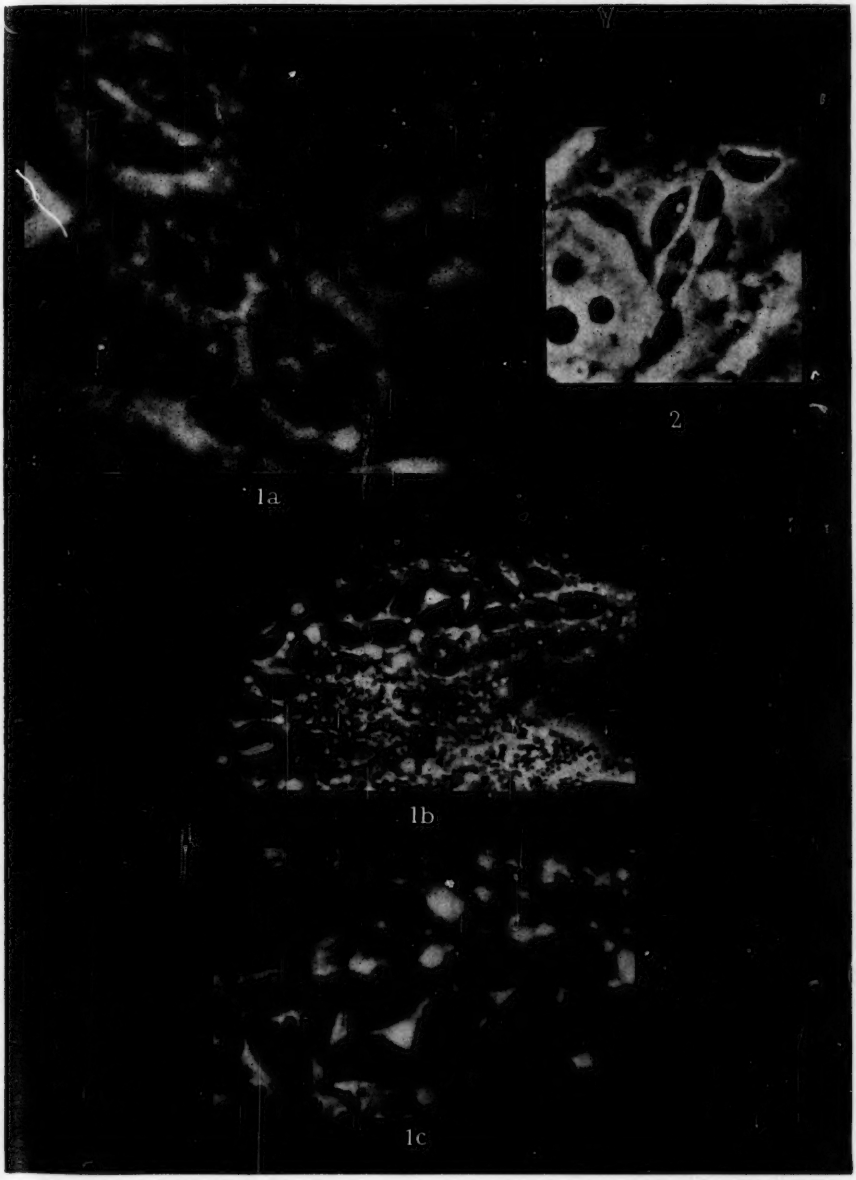
FIG. 3*a*–3*d*.—Toxoplasma parasite dividing in a vacuole. From Fig. 3*a* to 3*b* the division of the parasite nucleus is completed. In Fig. 3*c* the nucleoli are again clearly visible and fission of the cytoplasm is taking place. In Fig. 3*d* separation of the 2 daughter parasites seems to be completed. ×3350.

FIG. 4*a*–4*k*.—The binary fission of a parasite in a human embryonic kidney cell is followed. In Fig. 4*a* the fission has begun and in Fig. 4*k* the separation of the 2 daughter cells is completed. During the separation the parasites are turning and untwisting. Thus the lower parasite in Fig. 4*a*, for example, gradually turns so that the pointed end is following a half-circle starting from left to right of the upper part of the vacuole. Note the irregular configurations formed during the process of separation. ×3330.

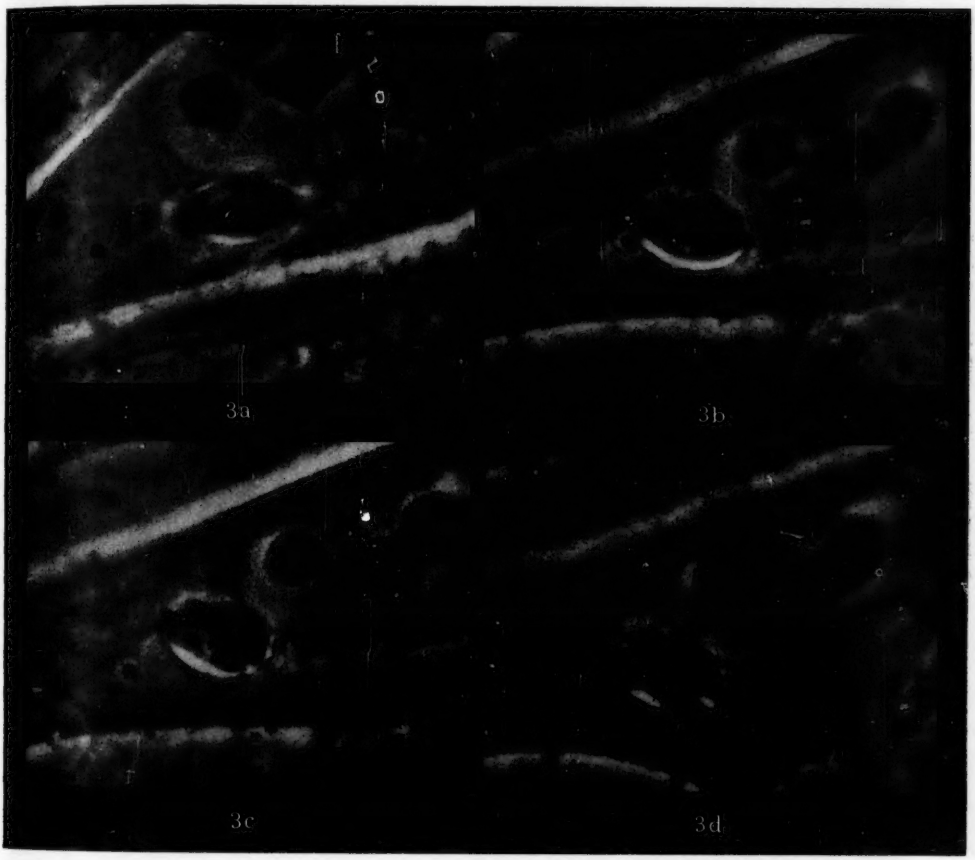
FIG. 5.—Two clones of parasites in vacuoles. The parasites of the clone at the left are arranged in a regular pattern. Note the membrane-like boundary of the vacuole. ×2600.

FIG. 6*a*—6*c*.—Rupture of a human embryonic kidney cell containing 2 clones of parasites. The parasites are arranged in a flower-like pattern in Fig. 6*a*. In Fig. 6*b* the regularity of the right hand clone is lost due to division of the parasites. In Fig. 6*c* the cell is bursting. The time lapse is 1·9 sec. between each of the pictures. ×520. Fig. 6*b* has been reproduced upside down accidentally.

FIG. 7.—A clone of parasites and 3 single parasites in a HeLa cell seen in the cytoplasm 24 hr. after infection. The clone of parasites is surrounded by a membrane-like boundary. Note the resemblance between this structure and the so-called pseudocyst. ×640.



Lund, Lycke and Sourander.


3a
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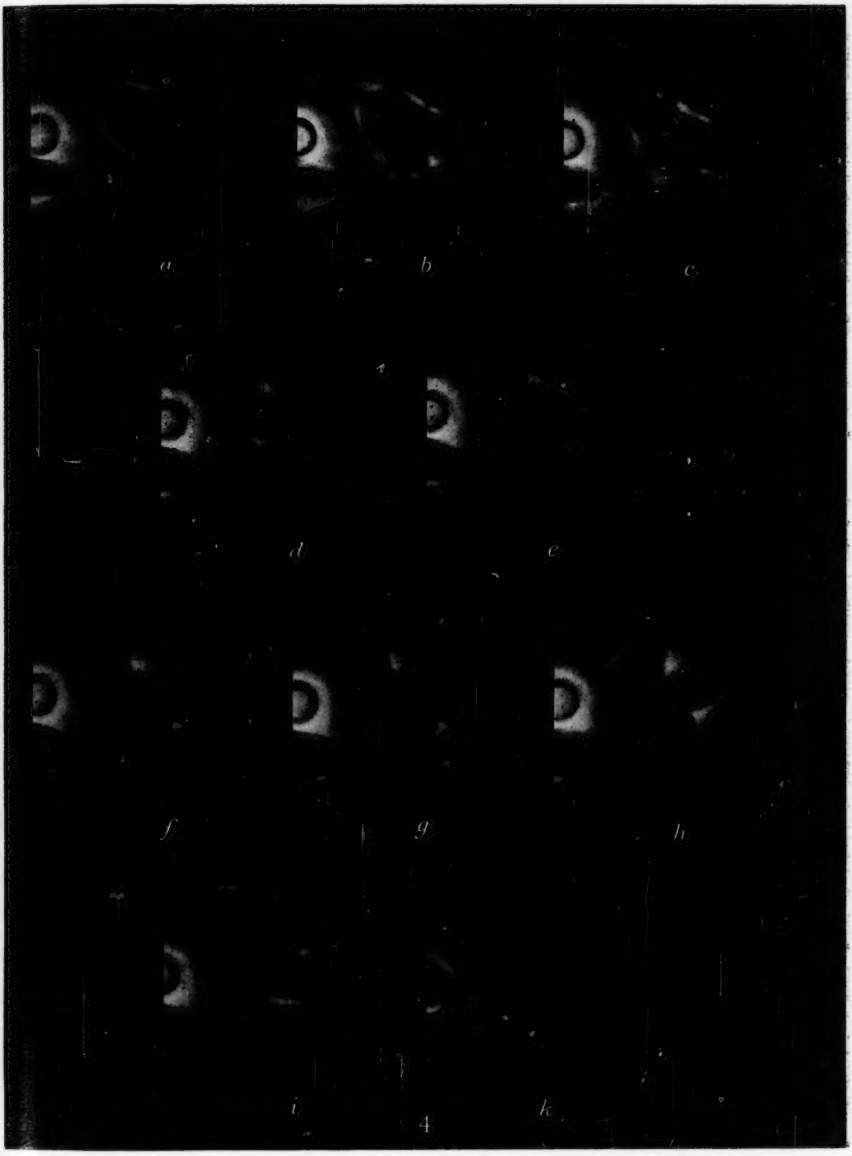

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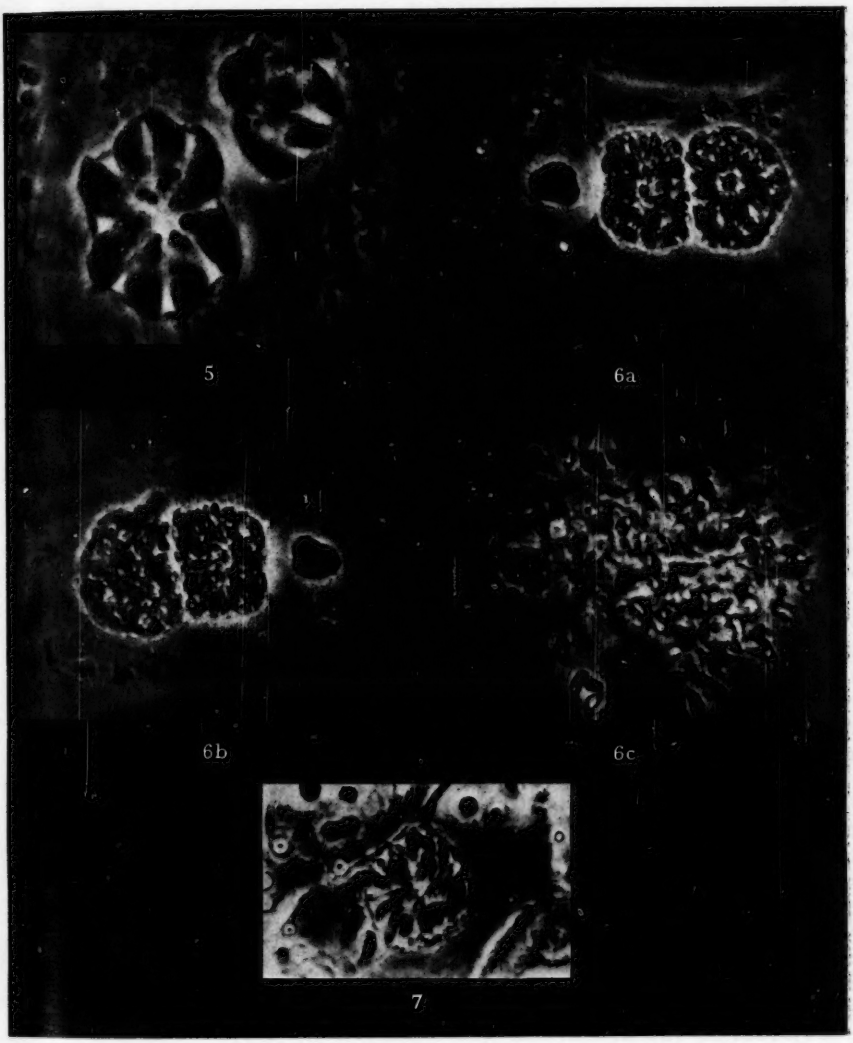
5
6a
6b
6c
7

After this liberation the cell was left with multiple perforations of its membrane.

Undoubtedly parasites will be liberated also when a cell degenerates. However lysis of cells is not due to parasitic infection but to other factors such as unfavourable culture conditions. Under such conditions also apparently enucleated cells containing parasites sometimes may be found. If the term terminal colony is used to describe the persisting clones in such devitalized cells then the term seems inadequate in describing a stage in the life cycle of toxoplasma.

So far, our observations of toxoplasma parasites in cell cultures, maintained even up to 3 months, have yielded no evidence for a vegetative form. In this connection it should be emphasized that large clones (Fig. 7) may show a striking resemblance with the so called pseudocysts in tissue sections from organs of toxoplasma infected animals and human patients.

Guimarães and Meyer did not observe cell rupture until 6 weeks after establishment of infected cultures. According to Cross and Anigstein (1949) cell rupture may occur in about 24 hr. after infection. Chernin and Weller (1957) using various types of tissue culture from man, monkey and mouse, observed cell rupture 6–7 days after inoculation. In our experiments rupture of human kidney cells was usually observed 24 hr. and rupture of HeLa cells 48 hr. after infection. Evidently, the variations reported must be due to cell and culture methods employed.

Movements of toxoplasma was first reported by Laveran. Guimarães and Meyer described the movements as undulating. Westphal described 2 types of movements, a circular movement of the entire parasite and a rotating movement of its pointed end. Also Manwell and Drobeck (1953) reported several types of movements. The speed and character of motility varied in different preparations. They observed also movements of the parasites within the host cell. Despite these observations, the motility of toxoplasma still seems to be a controversial subject (for reference see Thalhammer, 1957). Guimarães and Meyer were the first to report active penetration of the toxoplasma into cells. The penetration required 30–60 min. according to Schmidt-Hoensdorf and Holz. Pulvertaft, Valentine and Lane (1954) suggested 2 different ways in which parasites gain entrance into cells, active invasion or passive ingestion. Actively penetrating parasites did not multiply but were lethal to the cell whereas the ingested ones were the proliferating forms.

Our cinematographic observations clearly show the active movements of parasites liberated by cell rupture. These movements showed the same pattern as described by Westphal. Principally the movements seem to occur in the direction of the long axis of the parasite but the curved shape of the parasite makes variation in movements possible. The remarkable rate with which the parasites penetrate into new cells, observed immediately after cell rupture, must be a result of the active movements of the toxoplasmas. A few minutes after cell rupture active movements of extracellular parasites are no more detectable. Then it also takes longer time before the parasites are found intracellularly. The reduced motility of parasites in peritoneal exudates may also explain why a relatively long interval is required for primary infection of a cell culture with mouse exudates.

The internal structure of the toxoplasma parasite has been described by several electron microscopists. The most instructive studies are those published by Gustafson *et al.* (1954) and Ludvík (1956). Some of the internal structures were also discernible in the living toxoplasma with the phase contrast technique applied by us.

SUMMARY

A cinematographic study was made on cultured cells infected with *Toxoplasma gondii*. Details of the parasite multiplication resulting in binary fission were observed. Clones were formed by repeated division of parasites. The final event in parasitization was rupture of the host cell. This followed immediately after a division of parasites. Parasites of different generations were of equal size. Liberated parasites were actively moving, penetrated into new cells within few seconds and underwent a subsequent cycle of multiplication. The observations reported were discussed in relation to previous literature.

The skilful technical assistance of Mrs. Edith Johannsen is gratefully acknowledged.

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ENHANCEMENT OF GROWTH OF *PASTEURELLA PESTIS* AND OTHER BACTERIA IN SERUM BY THE ADDITION OF IRON

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Received for publication March 1, 1961

STRAINS of *Pasteurella pestis* which are fully virulent for mice form pigmented colonies when incubated at 28° on a suitable medium (Jackson and Burrows, 1956*a*). Non-pigmented mutants of virulent strains are markedly less virulent; this is associated with greatly reduced multiplication in the mouse. If, however, iron compounds are injected with these mutants, multiplication and lethal effects resemble those of fully virulent strains (Jackson and Burrows, 1956*b*).

The mechanism of the iron effect has been further investigated by studying growth in serum.

MATERIALS AND METHODS

Strains.—The strains of *Past. pestis* which were most extensively studied were M3 and M7; these have been described in a previous publication (Jackson and Burrows, 1956*a*).

The strains of *Pasteurella pseudotuberculosis*, PB1/+ and PB1/−, have been recently described by Burrows and Bacon (1960).

Strains of other bacteria were obtained from the culture collections held in this establishment.

Precautions against contamination with iron.—Pyrex glass vessels were used throughout. Stainless steel needles were used for collecting blood, in connection with silicone rubber tubing (manufactured by Esco (Rubber) Ltd., 2, Stothard Place, Bishopsgate, London, E.C.2) The water used for all purposes was distilled and passed through Amberlite Monobed Resin MB-1. Before sterilization, all apparatus was soaked for at least 24 hr. in a dilute solution of ethylenediamine-tetra-acetic acid (0·1 g. of the disodium salt dissolved in one litre of water) and then thoroughly rinsed in water.

Serum.—The difficulty in obtaining a convenient volume of mouse serum is obvious, and most of the experiments were carried out with human serum. Blood was obtained from 2 male volunteers, one belonging to Blood Group A, Rhesus negative, the other to Group O, Rhesus positive. Specimens of serum were prepared by immediate centrifugation (5 min. at 1,750 *g*) of about 250 ml. of blood taken from an arm vein. The red cells sedimented before coagulation occurred; the fibrin clot, compressed by centrifugal force, was found above the layer of red cells. A good yield of non-haemolysed serum was obtained.

For comparison, a specimen of mouse serum was prepared from the pooled blood collected by heart puncture from 12 albino mice (weight 50 g.) of the Porton strain. About 1 ml. of blood could be obtained from each mouse. The mouse blood coagulated very rapidly, but serum was satisfactorily expressed and separated on centrifugation after cutting the clot; this specimen was very little haemolysed in contrast to our usual experience with heart blood collected after opening the thoracic cavity of the mouse under anaesthesia.

Culture methods.—Bacteria to be tested were grown overnight on tryptic meat agar slopes. It was found that inocula of *Past. pestis* grown at either 28° or 37° gave similar results in serum; *Pasteurellae* for testing were, therefore, grown at 28°, at which temperature growth

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on tryptic meat agar is more consistent than at 37°. Other species to be tested were grown on the same medium at 37°.

Inocula were prepared by suspending the bacteria in phosphate buffer (pH 7·4) at a density of 10^8 organisms per ml., and then diluting 10-fold in serum. A standard small drop (1/36 ml.) of this serum suspension was used to inoculate 1 ml. of serum, which was then divided into aliquots of 0·5 ml. for the growth tests. The initial population was thus *ca.* 0·3 million per ml. Concentrations of additive solutions were chosen so that 1/36 ml. added to 1 ml. or 0·5 ml. of test serum gave the required test concentration. In first defining the iron effect and in subsequent controls, the standard addition was ferrous ammonium sulphate, to give a test concentration of 10^{-4}M (5·6 μg. of ferrous iron per ml.). In imitation of physiological conditions, serum cultures were incubated at 37°, in tubes sealed with vaccine caps, the gas space of the tubes (about 4 ml.) being filled with an atmosphere of 95 per cent air and 5 per cent CO_2. The tubes were gently rotated at approximately 67 rpm throughout incubation, with their axes tilted at 45° to the vertical.

Measurement of growth.—Changes in the numbers of viable bacteria were measured by diluting and plating out on solid medium (tryptic meat agar, supplemented for *Past. pestis* with 0·01 per cent haemin incubated at 28°) using the technique of Miles and Misra (1938).

RESULTS

There was no significant difference in the behaviour of strains M3 and M7 of *Past. pestis* in human and mouse serum; both grew to a limited extent in plain serum (serial sub-culture led to complete failure of growth in the second or third culture); both showed a marked increase in growth when ferrous iron was added to the serum. Similar results were obtained with human serum from either of the 2 volunteers of different blood groups.

TABLE I.—*Increase in Growth of* Pasteurella pestis *Strains M3 and M7, in Serum, on Addition of Ferrous Sulphate* (5·6 μg. Fe *per ml.*)

	Number of organisms (millions per ml.)				
	Strain M3		Strain M7		
Serum	Plain serum	Ferrous sulphate added	Plain serum	Ferrous sulphate added	Time from inoculation (hr.)
Human*	20	447	11	625	25·0
Human†	3	358	6	286	25·3
Mouse	14	1660	3	1071	21·5

* Donor was of Blood Group A (Rh negative).
† Donor was of Blood Group O (Rh positive).

Although it was soon evident that the difference in virulence between M3 and M7 was not due to a difference in their facility of growth in serum, the remarkable response to iron additions seemed worthy of further investigation.

Growth with different concentration of ferrous iron

Measurements of growth in greater concentrations of added ferrous iron (Table II, Expt. A) revealed that there was no further increase in growth beyond the level reached with 5·6 μgFe per ml. of serum.

When the concentration of ferrous iron added was reduced to less than 5·6 μg. per ml., it appeared that there might be a threshold between 1·4 and 2·8 μg.

TABLE II.—*The Effect of Other Ferrous Sulphate Concentrations or of Other Iron Compounds on the Growth of* Past. pestis, *Strains M3 and M7, in Human Serum*

Experiment	Iron additions in μg. per ml.	Iron additions as	Growth in million orgs. per ml. 24 hr. M3	24 hr. M7	42 hr. M3	42 hr. M7	48 hr. M3	48 hr. M7
A	0	Ferrous ammonium sulphate	23	6	9	6	11	4
	5·6	Ditto	351	436	725	453	725	614
	56	,,	259	87	259	344	367	481
	560.0	,,	342	257	120	217	306	271
B	0	—	17	5				
	1·4	,,	65	112				
	2·8	,,	536	345				
	5·6	,,	199	200				
	28·0	,,	432	387				
C	0	,,	5	1				
	5·6	,,	239	178				
	5·6	Ferric chloride	269	164				
D	0	—	20	11				
	6	Haemin	31	23				
	ca. 10	Lysed mouse erythrocytes	26	49				
	5·6	Ferrous ammonium sulphate	448	626				

ferrous iron per ml., above which growth was independent of ferrous iron concentrations (Table II, Expt. B). This threshold concentration may be correlated with the unsaturated iron-binding capacity of the serum, which was approximately 2·2 μg. of iron per ml. of serum (measured by the method of Schade, Oyama, Reinhart and Miller, 1954). It appears that, for maximum growth in serum, enough iron must be added to exceed its iron-binding capacity. This conclusion must be made with reservation since the population observed with 1·4 μg. ferrous iron per ml. was larger than that in plain serum.

Effects of other iron compounds on the growth of M3 and M7 in serum

At concentrations of 5·6 μg. of iron per ml. of serum, ferric iron had a similar effect to ferrous iron in promoting the growth of M3 and M7 (Table II, Expt. C). However, haemin had no significant effect, nor did a lysate of mouse erythrocytes, prepared by separating the red cells from serum and re-suspending them in a similar volume of sterile water (Table II, Expt. D). Evidently iron in haemoglobin or haemin is not available to either strain.

Failure of other metal ions to promote the growth of M3 and M7 in serum

The specificity of the effect of iron is shown in Table III. M3 and M7 appeared to be indifferent to the presence of other metal ions at comparable concentrations apart from zinc, which seemed to be slightly inhibitory.

TABLE III.—*The Effect of Other Metals on the Growth of M3 and M7 in Human Serum*

Metal salt added	Concentration of metal ion introduced (μg. per ml.)	Number of organisms after incubating for 26 hr. (millions per ml.)	
		M3	M7
None	—	27	41
Ferrous sulphate	5·6	444	484
Manganese sulphate	17·1	48	47
Cobalt sulphate	11·7	48	[illegible]
Nickel chloride	16·2	20	[illegible]
Copper sulphate	15·7	28	[illegible]
Zinc sulphate	12·6	2	4
Magnesium sulphate	6·2	19	51
Calcium chloride	13·0	36	12

The growth in serum of other strains of Past. pestis *and of* Past. pseudotuberculosis

The behaviour of strains of *Past. pestis* other than M3 and M7 was examined (see Table IV). All responded similarly when ferrous ion was added to serum. It is notable that there was no significant difference between the growth of virulent and avirulent strains, nor between pigmented and non-pigmented strains.

The strains PB1/+ and PB1/− of *Past. pseudotuberculosis* also grew well in serum containing added iron. It appears, indeed, that these organisms did not survive in serum for 24 hr. unless iron was added.

TABLE IV.—*The Growth of Other Strains of* Pasteurella *in Serum, With and Without Added Iron*

Experiment	Strain	Pigmentation	Virulence for mice	Growth in million orgs. per ml.	
				−Fe	+Fe
Controls	M3	+	V	3	358
	M7	−	RV	3	258
A. Other strains of *Past. pestis*.	MP6	+	V	4	130
	M14	−	RV	12	139
	TS	+	AV	9	195
	T14	−	AV	1	141
Controls	M3	+	V	5	239
	M7	−	RV	1	178
B. Strains of *Past. pseudotuberculosis*	PB1/+	+	AV	0·03	127
	PB1/+	+	AV	0·03	324

V = LD_{50} 10 organisms
RV = LD_{50} 10^3–10^7 organisms
AV = LD_{50} 10^8 organisms
} Intraperitoneal injections.

Effect of added iron on the growth of other bacterial species in serum

Some other species subjected to test behaved as shown in Table V. On the one hand, a most impressive response to iron was observed with a virulent strain of *Salmonella typhimurium*, while on the other the inability of strains of *Shigella*

and *Escherichia coli* to multiply in serum was unaffected by the presence of iron. A strain of *Staphylococcus aureus* was stimulated by iron, but not to the same extent as *Pasteurellae* and the strain of *Salmonella*.

TABLE V.—*Effect of Added Iron on the Growth of Other Bacterial Species in Human Serum*

Species and strain	Number of organisms after incubation for 27 hr. (millions per ml.)	
	Plain serum	Ferrous sulphate added (5·6 μg. Fe per ml.)
Salm. typhimurium LT2	<0·3	828
Sh. dysenteriae Bucharest (Smooth)	<0·3	<0·3
Sh. dysenteriae Bucharest (Rough)	<0·3	<0·3
Esch. coli. B.	<0·3	<0·3
Staph. aureus Oxford H (NCTC 6571)	0·4	33
Past. pestis M3	1	251
Past. pestis M7	1	281

In all cases the initial population was approximately 0·3 million organisms per ml., but the Shigella strains and the *Esch. coli* were very rapidly agglutinated.

In some cases, no colonies were observed at the lowest dilutions plated out (after incubation for 27 hr.); a maximum limit for the viable count was calculated. It is unlikely that the organisms in these tests had multiplied to any significant extent, although the occurrence of agglutinations was a complicating factor.

DISCUSSION

This investigation has not revealed the basis for the difference in virulence between strains M3 and M7 of *Past. pestis*. The difference in their ability to multiply *in vivo* remains unexplained.

However, the great increase of growth in serum in the presence of added iron makes it plausible that the similar growth response of M7 in mice injected with iron salt (Jackson and Burrows, 1956*b*) may be due to the stimulation of bacterial growth rather than to interference with defence mechanisms of the host.

It is possible that other bacterial species may be inhibited from multiplication *in vivo* by inability to acquire iron.

Presumably iron is readily assimilated *in vivo* by virulent pathogens. The recent work of Cavanaugh and Randall (1959) in the multiplication of *Past. pestis* within mononuclear leucocytes suggests the speculation that assimilable iron may be found within the cells of the host animal.

It would be interesting to determine whether added iron may influence the behaviour of organisms in tests to determine the bactericidal action of sera *in vitro*.

SUMMARY

The growth of *Past. pestis* in human serum at 37° was very limited unless iron was added, in which case growth was very greatly enhanced. No differences

found in the behaviour of different strains were sufficient to account for differences in virulence. Salts of other metals of known biological importance were ineffective.

Among other strains of bacteria tested, *Past. pseudotuberculosis* and *Salm. typhimurium* also showed a very big response to iron added to serum; *Staph. aureus* responded to a lesser extent, but *Sh. dysenteriae* and *Esch. coli* failed to grow with or without added iron.

We wish to express our gratitude to Dr. D. W. Henderson, for his interest and encouragement, to Dr. T. W. Burrows for many valuable discussions and for detailed consideration of the text, and to Mr. J. M. Hutchinson, Mr. S. J. Lister and Mr. J. S. M. Mayo for giving blood for our experiments.

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MALFORMATIONS IN FOETAL RATS INDUCED BY THE PYRROLIZIDINE ALKALOID, HELIOTRINE

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Received for publication March 6, 1961

THE pyrrolizidine alkaloids are of considerable theoretical interest because of the many peculiarities of their toxic action. In addition, their occurrence in numerous species of 3 important plant families, including the genera *Senecio*, *Crotalaria* and *Heliotropium*, makes them a hazard of practical importance to domestic animals and even to man in many parts of the world.

Ingestion of toxic plants causes a slowly progressive, ultimately fatal, liver disease in sheep, cows and horses (Bull, 1955 ; Bull, Dick, Keast and Edgar, 1956) and contamination of edible cereals with seed of *Heliotropium lasiocarpium* was found to be associated with human liver disease in central Asia during the years 1931–45 (Bourkser, 1948). Medicinal administration to man also occurs, with potentially dangerous consequences (Schoental, 1959).

A single high dose of the purified alkaloid heliotrine, in rats, causes extensive acute liver necrosis, initially centrilobular, the male being more susceptible than the female (Bull, Dick and McKenzie, 1958 ; Christie, 1958*a*). The administration, of repeated small doses of the purified alkaloids heliotrine and lasiocarpine to rats causes progressive atrophy of the liver, accompanied by gross cytological aberration (Bull and Dick, 1959). The development of chronic progressive liver disease in rats after a single moderately high dose of a pyrrolizidine alkaloid, and acceleration of the toxic effect when dietary protein is restricted, were reported by Schoental and Magee (1957). Malignant hepatomata have also been produced in rats with moderately high doses of pyrrolizidine alkaloids administered intermittently (Cook, Duffy and Schoental, 1950 ; Schoental and Head, 1957). The acute toxic effect of heliotrine in rats was found to be associated with disorganization of the tricarboxylic-acid respiratory cycle of the liver cells due to deficiency of mitochondrial diphosphopyridine nucleotide (Christie, 1958*b*).

All the preceding observations point to a direct action by the toxic compounds on hepatic parenchymatous cells, but there is evidence of additional effects. The existence of a toxic action on blood vessels has been postulated (Selzer and Parker, 1951 ; Selzer, Parker and Sapeika, 1951 ; Bras, Berry and György, 1957 ; Hill, Stephenson and Filshie, 1958 ; Bull *et al.*, 1958 ; Christie, 1958*a* ; Bull and Dick, 1959). Pyrrolizidine alkaloids have also been described as acetyl-choline antagonists (McKenzie, 1958) and as neuromuscular blocking agents (Gallagher and Koch, 1959).

Heliotrine and related alkaloids have been shown to have a powerful mutagenic action in the fruit-fly *Drosophila melanogaster* (Clark, 1959). Considerable increases in sex-linked recessive lethals, causing apparently irreversible blocks in immature

germ-cell maturation, lead to degrees of sterility which vary with the concentrations of alkaloid used. The natural occurrence of these chemical mutagens in plants raises the possibility of their continuous action after ingestion, so that livestock not fatally affected by the hepatotoxic action might suffer degrees of genetic deterioration.

Administration of the alkaloids lasiocarpine and retrorsine to lactating rats, in doses having no apparent effects on the mothers or on lactation, caused fatal liver lesions in the young (Schoental, 1959). Thus, infantile liver disease might result from contamination of human or cow's milk.

In view of these observations, the possibility that teratogenic effects might occur led to the following experiments.

MATERIALS AND METHODS

Solutions of heliotrine were freshly prepared from weighed amounts of purified crystalline alkaloid added to distilled, deionized water, and brought into solution by addition of sufficient HCl to adjust pH to 7·3.

Nulliparous inbred female Sprague-Dawley rats from this Department, and hooded rats from the Division of Animal Health, Commonwealth Scientific and Industrial Research Organization, Parkville, Victoria, each received a single intraperitoneal injection of heliotrine solution during gestation. To determine the optimum dose for teratogenic effects the following range was used : 15, 20, 30, 40, 50, 100, 150, 200 and 300 mg. heliotrine/kg. maternal body weight at the time of injection.

Approximately one half of the gestations in the Sprague-Dawley rats resulted from brother-sister unions, the remainder (including those of all hooded rats), from random matings. Mating periods were 1–2 hr. in one quarter of the cases, 6–8 hr. in one half, and longer than 8 hr. in the rest.

Each gestation was terminated surgically between 20 and 20½ days following fertilization, which was assumed to have occurred some 10 hr. after the mid-point of the mating period during which insemination occurred (Huber, 1915 ; Witschi, 1956 ; Dalcq, 1957). At laparotomy, foetal swellings in the cornua were counted, and mechanically stimulated to elicit movements. The whole uterus was then removed and fixed in 10 per cent formol saline prior to detailed macroscopic and microscopic examination. Foetal carcasses, after removal of viscera, were routinely cleared in 2 per cent KOH before selective skeletal staining with the dye Alizarin Red S by the method of Dawson (1926). The skeleton of a normal rat foetus stained in this way is illustrated in Fig. 1.

Maternal ovaries were retained for counts of corpora lutea for correlation with implantation sites. Thirty-two Sprague-Dawley and 21 hooded rats were used as controls.

RESULTS

Rats given less than 100 *mg. heliotrine/kg. maternal body weight*

The group (Table I) included 4 Sprague-Dawley and 2 hooded rats, each of which received a single injection of heliotrine (Table VI). All animals survived injection, but as the dose of the alkaloid was increased, temporary reductions of maternal weight following injection became more obvious.

Litters exposed to less than 50 mg. heliotrine/kg. maternal weight were normal. Of the 2 hooded rats which received 50 mg./kg., one showed complete resorption of a litter of 12. In this case, resorptions were so advanced as to suggest intrauterine death before injection on the 10th day of gestation. The remaining litter included 8 living young. Although weights and lengths of its members were well below normal, malformations were not detected.

TABLE I.—*Gestation at 20 Days in Rats Following Single Maternal Intraperitoneal Injections of Heliotrine in Concentrations Below 100 mg./kg.*

Rat No.	Injection: Dose (mg./kg.)	Injection: Time (day)	Weight gain (g.)	Number of corpora lutea	Foetal implantations	Number of resorptions	Surviving young: Number	Surviving young: Av. wt. (g.)	Surviving young: Av. lgth. (cm)	Surviving young: Malformed
				Sprague-Dawley Rats						
1	15	12·0	75	15	13	2	13	3·4	3·6	0
2	20	13·0	60	12	11	1	10	3·6	3·6	0
3	30	13·0	75	—	13	4	9	3·3	3·4	0
4	40	13·0	—	—	—	—	10	—	—	—
	Controls		73	13	10·6	2·3	8·2	3·5	3·4	0
				Hooded Rats						
5	50	9·0	45	11	10	2	8	2·3	2·8	0
6	50	10·0	15	12	12	12	0	—	—	—
	Controls		58	10·9	9·8	0·7	9	3·3	3·4	0

Rats given 100 mg. heliotrine/kg. maternal body weight

Each of 4 Sprague-Dawley and 4 hooded rats (Table II) received a single injection at the phase of gestation indicated, and all survived injection. In two instances, outward evidence of gestation did not follow insemination. In one of these cases, a cystic tumour of one ovary was found at laparotomy.

TABLE II.—*Gestation at 20 Days in Rats Following Single Maternal Intraperitoneal Injections of 100 mg. Heliotrine/kg.*

Rat No.	Day of injection	Weight gain (g.)	Number of corpora lutea	Foetal implantations	Number of resorptions	Surviving young: Number	Surviving young: Av. wt. (g.)	Surviving young: Av. lgth. (cm.)	Surviving young: Malformed
				Sprague-Dawley Rats					
7	6·0	30	—	11	9	2	2·3	3·2	0
8	8·0	25	11	11	11	0	—	—	—
9	11·0	65	11	11	2	9	2·8	3·3	0
10	12·0	No gestation							
	Controls	73	13	10·6	2·3	8·2	3·5	3·4	0
				Hooded Rats					
11	9·0	15	11	10	7	3	2·3	2·9	0
12	10·0	30	8	8	8	0	—	—	—
13	12·0	40	12	9	1	8	1.7	2·7	8
14	13·0	No gestation (cystic tumour of one ovary)							
	Controls	58	10·9	9·8	0.7	9	3·3	3·4	

All other females of the group showed subnormal gestational weight gains and, in all but one case (Rat 9) injection was followed by temporary loss of weight. Intra-uterine mortality was greater than in the preceding group (Table I). Of 6 litters produced, 2 were totally resorbed, and high resorption rates were shown in two others.

All surviving young were of subnormal size and weight. All members of one litter (Rat 13, exposed at 12·0 days gestation) in which foetal dwarfism was most conspicuous, showed retarded skeletal development, and in each case the ribs showed anomalous twisting of their para-vertebral segments (Fig. 2). No other abnormality was detected in any of these litters.

TABLE III.—*Gestation at* 20 *Days in Rats Following Single Maternal Intraperitoneal Injections of* 150 *mg. Heliotrine/kg.*

Rat No.	Day of injection	Weight gain (g.)	Number of corpora lutea	Foetal implantations	Number of resorptions	Surviving young: Number	Surviving young: Av. wt. (g.)	Surviving young: Av. lgth. (cm.)	Surviving young: Mal-formed
				Sprague-Dawley Rats					
15	9·0	25	13	13	13	0	—	—	—
16	10·0	10	10	11	11	0	—	—	—
17	11·0	No gestation							
18	11·0	,,	,,						
19	12·0	75	14	12	3	9	2·5	3·1	9
20	13·0	80	12	11	0	11	2·8	3·3	0
21	14.0	85	—	9	1	8	3·4	3·2	0
22	14·0	80	13	13	1	12	3·7	3·3	3
23	16·0	60	11	12	1	11	2·6	2·9	11
Controls		73	13	10·6	2·3	8·2	3.5	3·4	0
				Hooded Rats					
24	4·0	10	—	14	13	1	1·9	2·7	0
25	12·0	10	12	11	11	0	—	—	—
26	14·0	30	13	5	1	4	0·9	2·2	4
Controls		58	10·9	9·8	0·7	9	3·3	3·4	0

Rats given 150 *mg. heliotrine/kg. maternal body weight*

Each of 9 Sprague-Dawley and 3 hooded rats (Table III) received a single injection at the phase of gestation indicated. All survived injection, but in 2 cases (subsequently found to be suffering from advanced pneumonia) outward evidence of gestation did not follow.

Of the remaining 10 animals, 6 showed subnormal gestational weight gain, and almost all showed moderate loss of weight at some stage after injection. Intra-uterine mortality was comparable to that of the previous group. Of 10 litters produced, 4 (Rats 15, 16, 24 and 25) showed total or almost total resorption. Foetal dwarfism was conspicuous among survivors, and was extreme in the litter of Rat 26 in which foetal-placental weight ratios approximated 3 : 1 (normal 7 : 1 at 20 days gestation).

Malformations were found in 4 litters, exposed to heliotrine at 12, 14 (2 litters) and 16 days of gestation respectively. In 3 of these litters all members were affected. Anomalies were predominantly skeletal and, as in the previous group, the ribs were most commonly affected. Some degree of twisting of their para-vertebral segments was usual, but appearances varied considerably between and within litters, according to whether all, or only some ribs were affected, and also according to the extent to which they were calcified.

In general, rib deformities resembled those observed in the 100 mg./kg. group (Fig. 2), but were often associated with more extensive failure of calcification (Fig. 3). In some, the bilateral deformities were more or less equidistant from vertebral centres (Fig. 3), but this was not always the case (Fig. 4). In some foetuses there were no actual rib deformities, but merely localized and sharply defined uncalcified zones (Fig. 5). In other foetuses, ribs appeared normal but vertebral ossification was retarded or absent (Fig. 6).

Other anomalies noted in this group included hypoplasia of the lower jaw (Fig. 8) syndactyly (in 6 offspring of Rat 19) and small or absent thymus glands (in offspring of Rat 23).

TABLE IV.—*Gestation at* 20 *Days in Rats Following Single Maternal Intraperitoneal Injections of* 200 *mg. Heliotrine/kg.*

Rat No.	Day of injection	Weight gain (g.)	Number of corpora lutea	Foetal implantations	Number of resorptions	Surviving young: Number	Surviving young: Av. wt. (g.)	Surviving young: Av. lgth. (cm.)	Surviving young: Malformed
				Sprague-Dawley Rats					
27	3·0	No gestation							
28	10·0	30	—	6	6	0	—	—	—
29	12·0	40	15	15	12	3	0·8	1·9	3
30	13·0	90	11	11	1	12	2·9	3·2	1
31	13·25	40	Died on 18th day of gestation						
32	14·0	30	„ „ 19th „						
33	15·0	40	12	10	0	10	2·1	3·0	10
34	16·0	40	16	16	7	9	1·9	2·8	9
Controls		73	13	10·6	2·3	8·2	3·5	3·4	0
				Hooded Rats					
35	11·0	25	11	11	10	1	1·0	2·0	0
36	12·0	No gestation							
37	14·0	45	10	8	1	7	2·5	3·1	7
Controls		58	10·9	9·8	0·7	9	3·3	3·4	0

Rats given 200 *mg. heliotrine/kg. maternal body weight*

Of 8 Sprague-Dawley and 3 hooded rats in this group (Table IV), 2 (Rats 31 and 32) were found dead and partly eaten in late gestation, and in 2 others (Rats 27 and 36), outward evidence of pregnancy did not follow insemination. Of the 8 animals remaining, most showed subnormal gestational weight gain and quite marked, though sometimes only temporary, loss of weight after injection.

Intra-uterine mortality was comparable with that of the preceding groups. Of 8 litters produced, one was totally resorbed (Rat 28) and high rates of resorption occurred in 2 others (Rats 29 and 35). Foetal dwarfism was conspicuous in all but one litter, and was extreme in offspring of Rats 29 and 35 exposed on the 11th and 12th days of gestation.

Anomalies were found in each of 5 litters exposed to heliotrine at 12, 13, 14, 15 and 16 days of gestation respectively. In 4 of these all members were abnormal, and the remaining litter contained one defective foetus only. In general, malformations were much more gross in this than in preceding groups. Skeletal anomalies of the types illustrated (Figs. 2–6) were common, and were usually accompanied

by other serious defects such as hypoplasia of the lower jaw (Fig. 8) which was present in almost all offspring of Rats 33 and 34; hydroureter (Rat 31) and imperforate anus associated with rudimentary or absent tail (Fig. 10).

Hydrops of various degrees was conspicuous in offspring of Rat 33 (exposed at 15 days gestation) in which it often masked hypoplasia of the lower jaw. This occurred also in one foetus of Rat 34 (Fig. 11) in association with absent tail, hypoplastic urinary system and pericardial haemorrhage. The most anomalous litter was that of Rat 29 injected at 12·0 days. Only 3 of 15 original members survived to 20 days gestation. In each survivor (Fig. 12) extreme general oedema was associated with virtual absence of the lower jaw, cleft palate, anomalous extremities and tail, poor cerebral development, unilateral anophthalmia and cardiac defects.

EXPLANATION OF PLATES

FIG. 1.—The Alizarin Red S-stained skeleton of a normal rat foetus delivered surgically at 20 days' gestation. ×2.

FIG. 2.—The Alizarin Red S-stained skeleton of a rat foetus delivered surgically at 20 days' gestation following maternal intraperitoneal injection of 100 mg. heliotrine/kg. at 12·0 days (Rat 13). The specimen suffers from dehydration but shows limited vertebral and costal calcification and anomalous twistings of the paravertebral rib segments. ×3.

FIG. 3.—The Alizarin Red S-stained skeleton of a rat foetus delivered surgically at 20 days' gestation following maternal intraperitoneal injection of 150 mg. heliotrine/kg. at 12·0 days (Rat 19). Costal calcification does not extend beyond zones of twisting which are more or less equidistant from vertebral ossification centres. ×3.

FIG. 4.—The Alizarin Red S-stained skeleton of a rat foetus delivered surgically at 20 days' gestation following maternal intraperitoneal injection of 150 mg. heliotrine/kg. at 12·0 days (Rat 19). In this case calcification of the ribs sometimes extends beyond zones of twisting which are not equidistant from vertebral centres. ×3.

FIG. 5. The Alizarin Red S-stained skeleton of a littermate of the rat foetus illustrated in Fig. 4. In this instance the ribs are not twisted but some of them show limited uncalcified zones at various distances from vertebral centres. ×3.

FIG. 6.—The Alizarin Red S-stained skeleton of a rat foetus delivered surgically at 20 days' gestation following maternal intraperitoneal injection of 150 mg. heliotrine/kg. at 14·0 days (Rat 26). Two thoracic vertebral centres of ossification only are present. The ribs do not show the anomalies illustrated in Fig. 2–5. ×3.

FIG. 7.—Lateral aspect of the head of a normal rat foetus delivered surgically at 20 days' gestation. The upper and lower jaws are of similar lengths. ×4.

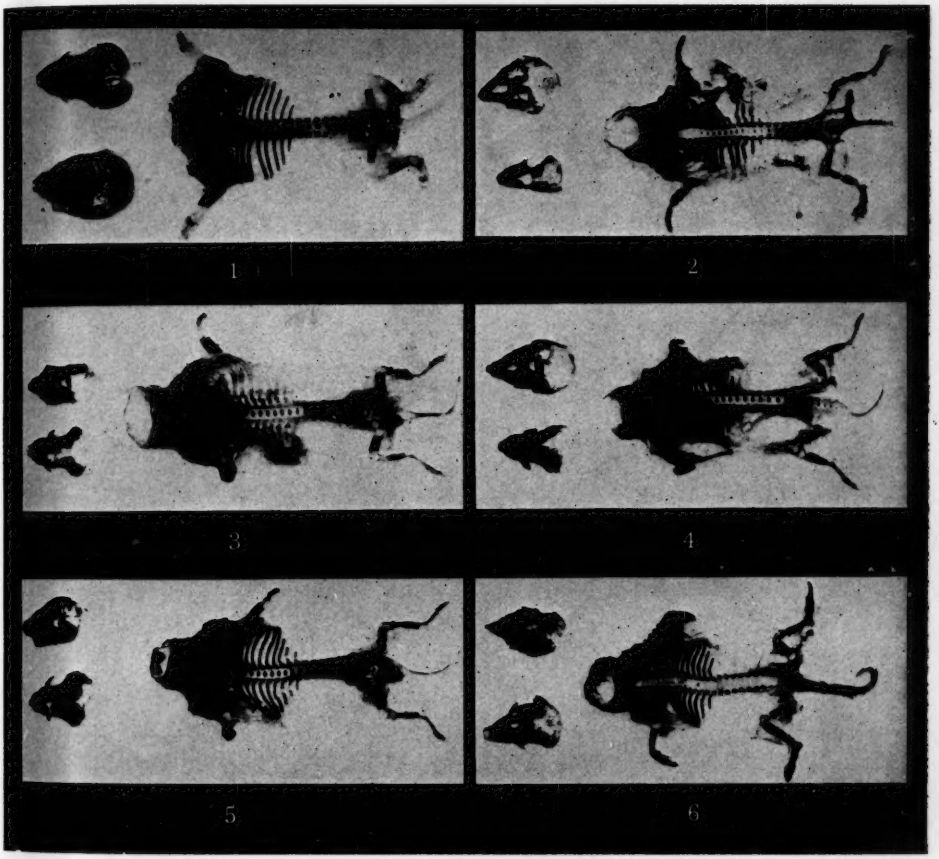
FIG. 8.—Lateral aspect of the head of a rat foetus delivered surgically at 20 days' gestation following maternal intraperitoneal injection of 200 mg. heliotrine/kg. at 15 days (Rat 33). The lower jaw hypoplasia shown is similar to that observed following maternal irradiation at 12 days 2 hr. gestation (Green, 1960). ×3·5.

FIG. 9.—The forepaw of a rat foetus delivered surgically at 20 days' gestation following maternal intraperitoneal injection of 150 mg. heliotrine/kg. at 12·0 days (Rat 19). Instead of the normal 5 digits, 4 only are present, apparently as a result of fusion of the 3rd and 4th members. ×12.

FIG. 10.—Rudimentary tail (left) of a rat foetus delivered surgically at 20 days' gestation following maternal intraperitoneal injection of 200 mg. heliotrine/kg. at 16·0 days (Rat 34). This anomaly was associated with imperforate anus, hypoplasia of the lower jaw and of the urinary system. The genital tubercle is shown on the right. ×20.

FIG. 11.—A rat foetus delivered surgically at 20 days' gestation following maternal intraperitoneal injection of 200 mg. heliotrine/kg. at 16·0 days (Rat 34). In this instance extreme oedema was associated with absence of the tail, imperforate anus, general hypoplasia of the urinary system and pericardial haemorrhage. ×1·5.

FIG. 12.—One of the three surviving members of a rat litter delivered surgically at 20 days' gestation following maternal intraperitoneal injection of 200 mg. heliotrine/kg. at 12·0 days (Rat 29). In all three there was extreme generalized oedema, virtual absence of the lower jaw and gross deformities of extremities and tail. Other anomalies included cleft palate, poor cerebral development, unilateral anophthalmia and cardiac defects. ×1·5.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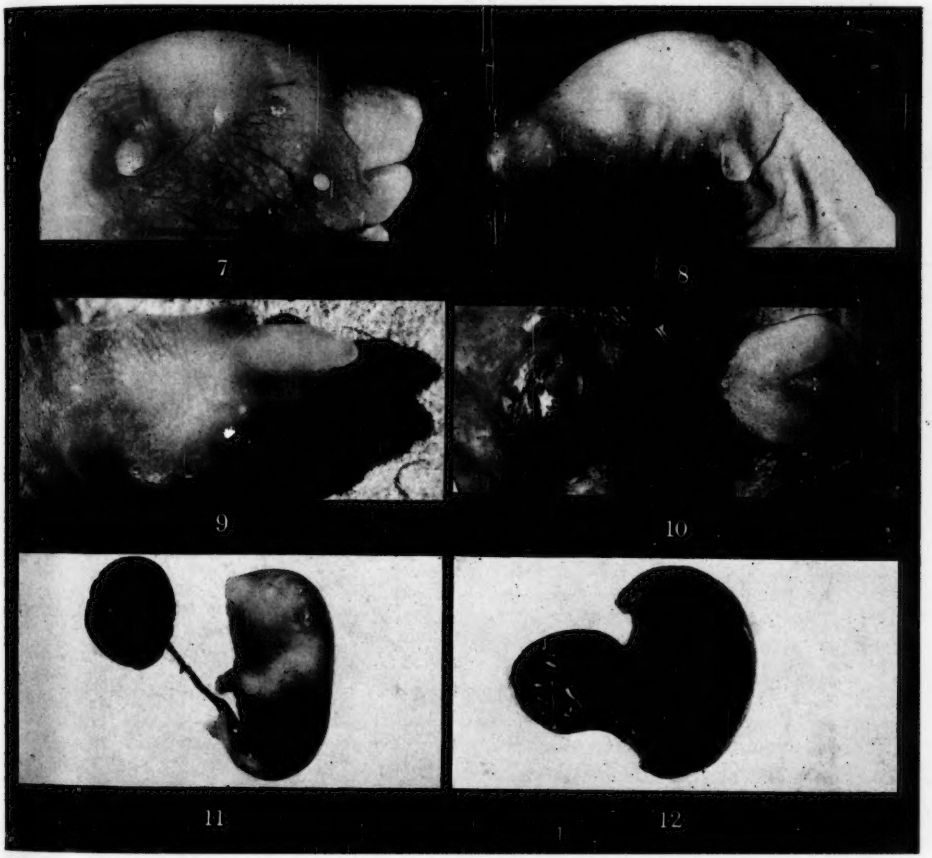
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10
11
12

TABLE V

Day of injection
9·0[1]
10·0[2]
12·0[3]
13·0[4]
13·0[5]
13·0[6]
6·0
8·0
9·0
10·0
11·0
12·0
12·0
13·0
4·0
9.0
10·0
11·0
11·0
12·0
12·0
13·0
14·0
14·0
14·0
16·0
3·0
10·0
11·0
12·0
12·0
13·0
13.25
14·0
14·0
15·0
16·0

TABLE VI.—*The Chief Abnormalities Observed in Foetal Sprague-Dawley and Hooded Rats Exposed to Heliotrine at Various Concentrations and Times of Gestation* (H)

Day of injection	Rat number	Surviving young	Number of young with anomalies of types or sites indicated													
			General			Visceral etc.					Musculo-skeletal					
			Dwarfism	Hydrops	Haemorrhage	Brain	Eye	Heart	Urinary	Anus	Palate	Mandible	Vertebrae	Ribs	Limbs	Tail
Less than 100 *mg./kg.*																
9·0[1]	5H	8	8	—	—	—	—	—	—	—	—	—	—	—	—	—
10·0[2]	6H	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
12·0[3]	1	13	—	—	3	—	—	—	—	—	—	—	—	—	—	—
13·0[4]	2	10	—	—	5	—	—	—	—	—	—	—	—	—	—	—
13·0[5]	3	9	—	—	2	—	—	—	—	—	—	—	—	—	—	—
13·0[6]	4	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—
100 *mg./kg.*																
6·0	7	2	2	—	—	—	—	—	—	—	—	—	—	—	—	—
8·0	8	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9·0	11H	3	3	—	—	—	—	—	—	—	—	—	—	—	—	—
10·0	12H	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
11·0	9	9	9	—	1	—	—	—	—	—	—	—	—	—	—	—
12·0	10	No gestation														
12·0	13H	8	8	—	1	—	—	—	—	—	—	—	—	8	—	—
13·0	14H	No gestation														
150 *mg./kg.*																
4·0	24H	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—
9.0	15	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10·0	16	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
11·0	17	No gestation														
11·0	18	,,	,,													
12·0	19	9	9	—	3	—	—	—	—	—	—	—	—	9	6	—
12·0	25H	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
13·0	20	11	8	—	—	—	—	—	—	—	—	—	—	—	—	—
14·0	21	8	2	—	—	—	—	—	—	—	—	—	—	—	—	—
14·0	22	12	—	—	—	—	—	—	—	—	—	—	—	3	—	—
14·0	26H	4	4	—	—	—	—	—	—	—	—	—	4	4	—	—
16·0	23	11	10	—	—	—	—	—	—	—	—	10	11	11	—	—
200 *mg./kg.*																
3·0	27	No gestation														
10·0	28	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
11·0	35H	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—
12·0	29	3	3	3	—	3	3	3	—	3	3	3	3	3	3	3
12·0	36H	No gestation														
13·0	30	12	12	—	—	—	—	—	2	—	—	—	—	1	—	—
13.25	31	Died on 18th day of gestation														
14·0	32	,, ,, 19th ,, ,,														
14·0	37H	7	7	—	—	—	—	—	—	—	—	—	3	2	—	—
15·0	33	10	10	10	—	—	—	—	—	—	—	10	7	7	—	—
16·0	34	9	9	—	1	—	—	—	1	4	—	8	3	3	—	2

[1] (50 mg./kg.).
[2] (50 mg./kg.).
[3] (15 mg./kg.).
[4] (20 mg./kg.).
[5] (30 mg./kg.).
[6] (40 mg./kg.).

TABLE V.—*Gestation in Rats Following Single Maternal Intraperitoneal Injections of 300 mg. Heliotrine/kg.*

Rat No.	Day of injection	Weight gain (g.)	Foetal implantations	Number of resorptions
			Sprague-Dawley Rats	
38	14·0	55	Died on 18th day of gestation	
39	15·0	15	„ „ 19th „ „	
			Hooded rats	
40	8·0	−25	4	4

Rats given 300 mg. heliotrine/kg. maternal body weight

Of the 3 rats which received this dose (Table V), 2 died during late gestation. The 3rd survived injection but showed total resorption of a small litter.

The chief anomalies observed in each group and their relationship to dose and time of exposure are summarized in Table VI.

The livers and other viscera of all dissected foetuses were examined histologically but the heliotrine-treated specimens were indistinguishable from controls.

DISCUSSION

Being exploratory in nature, this investigation was virtually confined to the 2nd week of gestation. However, it is clear from Table VI that induced abnormalities increased in frequency and severity as concentrations of the alkaloid were raised. Abnormalities followed administration of the drug on almost every day tested between the 4th and 16th, but in this small series they appeared slightly more commonly after injection on the 12th day. The results do not indicate any obvious difference in sensitivity to heliotrine between the 2 strains of rat used.

The commonest abnormality was dwarfism which appeared to be due to general retardation of intra-uterine development. It was observed in litters which survived injection at most of the times tested.

Next in frequency were musculoskeletal defects, particularly those affecting the ribs, which showed acutely angulated deformities and sharply demarcated bands of defective calcification. Variations in the severity and distribution of these component lesions produced a wide range of appearances, which varied not only between litters, but even between littermates.

Hypoplasia of the lower jaw also was frequent and was indistinguishable from the type observed following irradiation during gestation (Green, 1960) except that in the present instance it was accompanied by cleft palate only in its most extreme form.

Anomalous appearances of vertebral ossification centres were also noted. However, it is doubtful whether these should be regarded as specific anomalies rather than as extreme manifestations of retarded growth.

Although musculoskeletal anomalies predominated in the present experiments, it would be premature to conclude that this is a special feature of the action of heliotrine as opposed to other agents.

One of the problems set by the present observations is the mechanism by which these defects were produced. It is possible that the immediate cause of developmental anomalies of tissue defect or excess, produced by teratogenic agents whose action is not primarily genetic, may often be a disturbance of vasculature or of vaso-formative mesenchyme. It is therefore of interest that vascular damage is a probable component of the toxic action of many of the pyrrolizidine alkaloids, including heliotrine. Thus, the teratogenic effect may be more closely related to the vascular action than to the hepatotoxic, carcinogenic or mutagenic activities of these substances.

However, even if vascular disturbance is the immediate cause, the preliminary steps leading to it are likely to be intimately related to the metabolism of the alkaloid, of which little is known at present.

Apart from its intrinsic interest, the subject raises the practical question whether natural ingestion of material containing pyrrolizidine alkaloids might have deleterious effects on the reproduction of livestock, not only by mutational, but also by non-genetic disturbances of embryonic development.

Further investigations on questions arising from present findings are in progress.

SUMMARY

Malformations in foetal rats were observed, for the first time, to follow single maternal intraperitoneal injections of the pyrrolizidine alkaloid, heliotrine, administered chiefly during the second week of gestation. Abnormalities increased in frequency and severity as concentrations of the alkaloid were raised. Anomalies most commonly observed were general retardation of development and musculoskeletal defects involving especially the ribs. At high concentrations, hypoplasia of the lower jaw, indistinguishable from that produced by irradiation, was observed. In its severest forms, this was accompanied by cleft palate and other anomalies.

We wish to thank Dr. L. B. Bull and Dr. A. T. Dick of the Commonwealth Scientific and Industrial Research Organization, Animal Health and Research Laboratories, Parkville, Victoria, for their interest in this work and for supplies of materials.

The work was supported by a research grant from the Anti-Cancer Council of Victoria.

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THE IMMUNOLOGICAL REACTIVITY OF THE THYMUS

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Received for publication March 10, 1961

A NUMBER of previous studies (Fichtelius, 1957; Bjørneboe, Gormsen and Lundquist, 1947; Harris, Rhoads and Stokes, 1948; Maclean, Zak, Varco and Good, 1957; Askonas and White, 1956), indicate that the intact thymus gland shows very slight or no production of antibody in immunized animals and also that the gland does not undergo the histological changes observed in other lymphoid tissues as a result of the parenteral administration of antigens. In addition, Dixon, Weigle and Roberts (1957), observed a very slight transfer of secondary antibody response by thymic cells, and Thorbecke and Keuning (1953), reported a failure of antibody production in thymic explants from immunized animals and no histological changes in the intact thymus. The production of runt disease by thymic cells and the experiments of Stoner and Hale (1955) however, indicate that thymic cells possess full immunological competence.

This study was undertaken to clarify the reasons for these previous observations and more particularly, to relate them to the fact that the human thymus in myasthenia gravis may show changes suggesting an immune response.

MATERIALS AND METHODS

Animals

Male and female guinea-pigs and rats were employed in these experiments, the former animal being particularly suitable owing to the completely separated bi-lobed structure and cervical situation of its thymus gland. Young animals of about 400 g. (guinea-pig) and 130 g. (rat) were used.

Surgical procedures were carried out under nembutal and ether anaesthesia in the guinea-pig and ether anaesthesia in the rat. Direct injection of antigens at multiple sites into the thymus was performed in the guinea-pig with a tuberculin syringe and fine needle on one lobe of the exposed gland, the other being used as a control. TAB vaccine (Wellcome) diluted to 200 million orgs. per ml., and aluminium phosphate precipitated diphtheria toxoid (PAPT), 100 Lf or 6 Lf per ml. were employed as antigens; 0·2 ml. of antigen being injected. Aluminium phosphate mineral carrier was estimated at 1·5 mg. or 70 μg. per injection. Two series of injections were given at a 2 or 3 week interval, the animals being killed at 5–9 days after the last injection. Local thymic injury in the guinea-pig was produced by a cautery incision across the lower pole of one lobe of the gland. The opposite lobe was used as a control in the subsequent vital staining.

Vital staining was carried out on guinea-pigs and rats, both on the intact animal and in animals with thymic injury. A dose of 2 ml. of 2 per cent trypan blue (Revector) by subcutaneous injection or Evans blue (Gurr) was employed in the rat, and 8 ml. 1·5 per cent trypan blue per 400 g. body weight in the guinea-pig; the animals being killed 3–6 days after injection. Some animals were given a second injection after an interval of 2–3 days. In animals with thymic injury the dye was injected 30 min. after its infliction.

Localization of Injected Antigens and Antibody by the Fluorescent Antibody Technique : Polysaccharide and Protein Test Antigens

The following materials were injected by the external jugular vein in guinea-pigs and by the femoral vein in the rat. In guinea-pigs in which cautery injury had been inflicted on one lobe of the thymus, injection was made 10 min. after injury, and was normally made on the side opposite to that of the injured thymus lobe.

Streptococcus pneumoniae *type III capsular polysaccharide.*—Rats of 140 g. were injected with 10 mg. in 0·5 ml. of physiological saline. Guinea-pigs of 400 g. were injected with 12 mg. in 1·0 ml. of saline. These animals were killed 2 or 4 days after injection.

Diphtheria antitoxin.—Pepsin refined globulin prepared in the horse, provided by Burroughs Wellcome Ltd., 3300–5000 i.u. per animal.

Preparation of tissue sections

Localization of the capsular polysaccharide of streptococcus pneumoniae *type III.*—Portions of thymus, lymph nodes, spleen, and liver were fixed for from 18 hr. at room temperature to 48 hr. at 5° in a 65 per cent alcohol-10 per cent formalin mixture. Tissues were passed directly into 70 per cent alcohol, dehydrated and embedded in wax. Sections (5 μ) were floated out on warm 70 per cent alcohol and mounted avoiding all contact with water. After dewaxing in xylol and passage through absolute ethanol and 95 per cent ethanol to physiological saline, the sections were stained for pneumococcal polysaccharide by use of a single layer technique, with fluorescein isothiocyanate conjugate of the crude globulin fraction of a rabbit anti-pneumococcus (type III) serum. The conjugate was applied over the section and left in a moist chamber at room temperature for 30 min., followed by a wash for 10 min. in physiological saline buffer (0·15 M-NaCl : 0·01 M-phosphate pH 7·0).

Localization of injected diphtheria antitoxin and of antitoxin-containing cells in immunized animals.—Frozen sections (3 μ) were cut in a cryostat, thawed onto chromic acid cleaned slides, fixed with absolute methanol at room temperature (20°–22 °) for 15 min. and quickly washed in buffered saline. The sections were then exposed to a solution of diphtheria toxoid (500 Lf/ml.) for 30 min., washed in buffered saline for 5 min., exposed to horse diphtheria antitoxin labelled with fluorescein isothiocyanate and finally washed for 10 min. in physiological saline buffer. All fluorescent conjugates were absorbed twice with acetone lyophilized mouse liver powder. (100 mg. per ml.)

Controls for staining procedures with fluorescent antibody conjugates

Pneumococcus polysaccharide.—(i) Adjacent cryostat frozen sections were treated after fixation with a heterologous fluorescent antibody conjugate : fluorescein iso-thiocyanate conjugated rabbit anti-ovalbumin.

(ii) Adjacent cryostat frozen sections were treated with fluorescein conjugated anti-pneumococcus polysaccharide type III which had been absorbed with the calculated amount of homologous polysaccharide at 4° for 5 days followed by centrifugation to remove the antigen-antibody precipitate and checked to show no reaction with known polysaccharide containing sections.

Diphtheria antitoxin.—(i) Adjacent sections were treated after methanol fixation with fluorescein-isothiocyanate conjugated diphtheria antitoxin for 30 min., followed by a 10 min. wash in physiological saline-buffer. Such treatment would reveal diphtheria toxoid antigen and non-specifically stained structures.

(ii) Adjacent sections were treated after methanol fixation with a heterologous conjugate : fluorescein isothiocyanate rabbit anti-ovalbumin to reveal non-specifically stained structures.

Fluorescence microscopy.—The equipment previously described (White, 1960*a*), which employs ultraviolet radiation from a 20 amp. carbon arc source, filtered through 20 per cent. copper sulphate solution, was used. Visible radiation, was finally screened by a Corning 9840 2 mm. filter, with a Wratten 2B filter in the eyepiece.

RESULTS

Effect of Direct Injection of Antigens into the Substance of the Thymus Gland

In 6 guinea-pigs injected with multiple doses of 4×10^7 heat-killed typhoid-paratyphoid baccilli into the substance of the thymus, with an interval of 2–3

weeks and killed 5–9 days after the second series of injections, sections of the thymus showed two main histological changes. First, there were prominent confluent areas, often in close relation to the blood vessels or actually perivascular in situation, consisting of mature and immature plasma cells, and secondly, several lymphoid nodules with germinal centres (Fig. 1), apparently identical with those seen in other lymphoid organs, were present.

In the sections of 9 guinea-pigs injected with aluminium phosphate-precipitated diphtheria toxoid, large masses of amoeboid macrophages, with cytoplasm loaded with aluminium mineral salt particles were present in the medulla of the thymic lobules with adjacent areas of mature and immature plasma cells (Fig. 2). Under the fluorescence microscope these cytoplasmic particles fluoresced a bright greyish-blue (natural fluorescence). Segregated at the periphery of such masses, and often adjacent to or surrounding small blood vessels, and occupying the adventitia of these, were groups of antibody-containing mature and immature plasma cells (Figs. 3 and 4). The localization of such cells as revealed by the " sandwich technique ", which made use of fluorescein isothiocyanate-labelled diphtheria antitoxin after preliminary treatment of the section with dilute diphtheria toxoid, was checked by reference to adjacent frozen sections stained by Giemsa's method, and shown to correspond to groups of typical mature and immature plasma cells. The bright apple-green fluorescence was uniformly present throughout the cytoplasm or excluded a small juxta-nuclear area in the position of the Golgi element, and was also seen in some cells as irregular spots or rings within the dark oval of the nuclear area. It was apparent that the numbers of cells revealed as fluorescent antibody-containing cells were much less than the numbers of mature and immature plasma cells in corresponding areas of Giemsa-stained adjacent sections. Germinal centres were not seen in the thymuses of animals treated with aluminium phosphate-precipitated diphtheria toxoid. A surprising finding was bright fluorescence, indicating the presence of high concentrations of diphtheria antitoxin, in some of the Hassall's corpuscles. Control sections (see below) for the presence of homologous antigen or for the non-specificity of this reaction did reveal that these areas were to a slight extent non-specifically stained, but the difference between the test and control sections clearly indicated the presence of diphtheria antitoxin in high concentration at these sites.

Controls for the specificity of the staining reaction for diphtheria antitoxin were as follows :—First, sections stained by the same overall procedures as in the " sandwich technique " for diphtheria antitoxin, but omitting the treatment with dilute antigen (diphtheria toxoid) solution, resulted in no fluorescence in cells of plasma cellular morphology. Such control preparations showed very scanty cells with a cytoplasmic distribution of fluorescence in and among the masses of macrophages. From the granular nature of this fluorescence and the bi- or multi-lobed outlines of their nuclear shadows, these cells could be identified as polymorphonuclear leucocytes or eosinophils which had non-specifically localized the fluorescent globulin conjugate. The same controls showed that some of the cells in the centre of the medullary groups of macrophages had a faint apple-green cytoplasmic fluorescence, and that irregular green streaks and granules existed between such central cells. This was thought to indicate the presence of diphtheria toxoid antigen both within the cytoplasm of macrophages and extracellularly at these sites. The cytoplasm of surrounding macrophages fluoresced a bright greyish-blue owing to their content of aluminium mineral salt.

Secondly, sections stained with a heterologous fluorescein globulin conjugate (rabbit anti-ovalbumin) showed no localization to plasma cells, or to the central macrophages and sites of extracellular fluorescence seen in the above sections. However, the same scanty polymorphonuclear leucocytes or eosinophils as appeared in the above treatments also appeared in these control sections.

Vital Staining of the Normal and Injured Thymus

For vital staining of the normal and injured thymus, 20 rats and 13 guinea-pigs were employed. The results were similar in both species. Marked staining of the macrophages in the connective tissue capsule and septae of the thymus was seen (Fig. 5) and in the rat, well marked staining of the sinus cells of the small lymph nodes on the external surface of the thymus was present (Fig. 6). In some animals no definite staining of cells in the medulla or cortex of the thymus could be found. In others occasional granules of dye could be picked up in macrophages, usually situated at the cortico-medullary junction or at the periphery of the thymic cortex. These results could not be explained by an absence of reticulo-endothelial cells from the gland, as metallic impregnation methods for microglia (Fig. 7) showed a dense plexus of cells in the medulla resembling those seen in other lymphoid organs and previously classified by one of us (Marshall, 1953), as " metalophil cells ". It would appear that these cells account for a large number of the cells in the medulla of the thymus usually classified as " epithelial reticular cells ". The cells of the Hassall's bodies however, remained unstained by this metallic impregnation method.

In the guinea-pig thymuses which had received a cautery incision, marked vital staining was seen in macrophages in the medulla of the thymus in the region

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EXPLANATION OF PLATES

FIG. 1.—Germinal centre in medulla of thymus following direct injection of TAB vaccine. H. and E. ×114.

FIG. 2.—Plasma cells in medulla of thymus at edge of area of PAPT deposit. H. and E. ×684.

FIG. 3.—Fluorescence micrograph : antibody-containing cells in thymus at margin of deposit of PAPT-containing macrophages. Diphtheria antitoxin fluorescein conjugate. ×114.

FIG. 4.—Fluorescence micrograph : antibody containing cells similar to those in Fig. 3. Diphtheria antitoxin-fluorescein conjugate ×456.

FIG. 5.—Vitally stained cells in connective tissue septae of rat thymus. Note unstained thymic tissue. Trypan blue. Neutral red counterstain. ×85.

FIG. 6.—Vitally stained cells in lymph node on surface of rat thymus. Note unstained adjacent thymic tissue. Trypan blue. Carmine counterstain. ×100.

FIG. 7.—Metalophil reticulo endothelial cells in medulla of rat thymus. Weil-Davenport method for microglia. ×135.

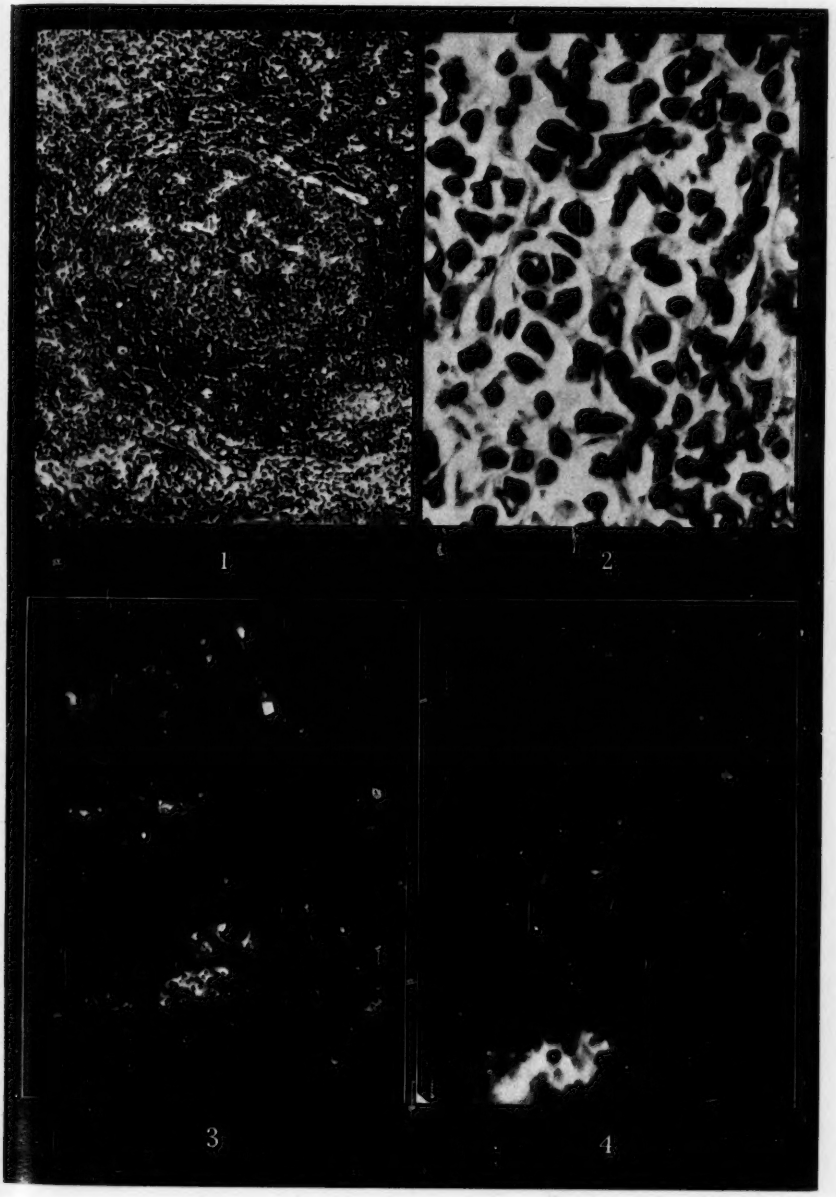
FIG. 8.—Marked vital staining of macrophages in medulla of thymus in region of cautery incision. Trypan blue. H. and E. ×315.

FIG. 9.—Fluorescence micrograph. Specific fluorescence denoting presence of pneumococcal polysaccharide in cells of rat thymic capsule, 48 hr. after injection.

FIG. 10.—Fluorescence micrograph. Marked specific fluorescence denoting presence of pneumococcal polysaccharide in lymph node on surface of rat thymus, 48 hr. after injection. Note fluorescent cells in adjacent thymic capsule and lack of staining in thymus. ×85.

FIG. 11.—Fluorescence micrograph. Pneumococcal polysaccharide in lumen of blood vessel in medulla of thymus. Non-specific fluorescence of Hassall's body at opposite side of figure. ×456.

FIG. 12.—Fluorescence micrograph. Numerous fluorescent polysaccharide-containing cells in region of cautery incision in guinea-pig thymus, 4 days after intravenous injection of pneumococcal polysaccharide. ×114.



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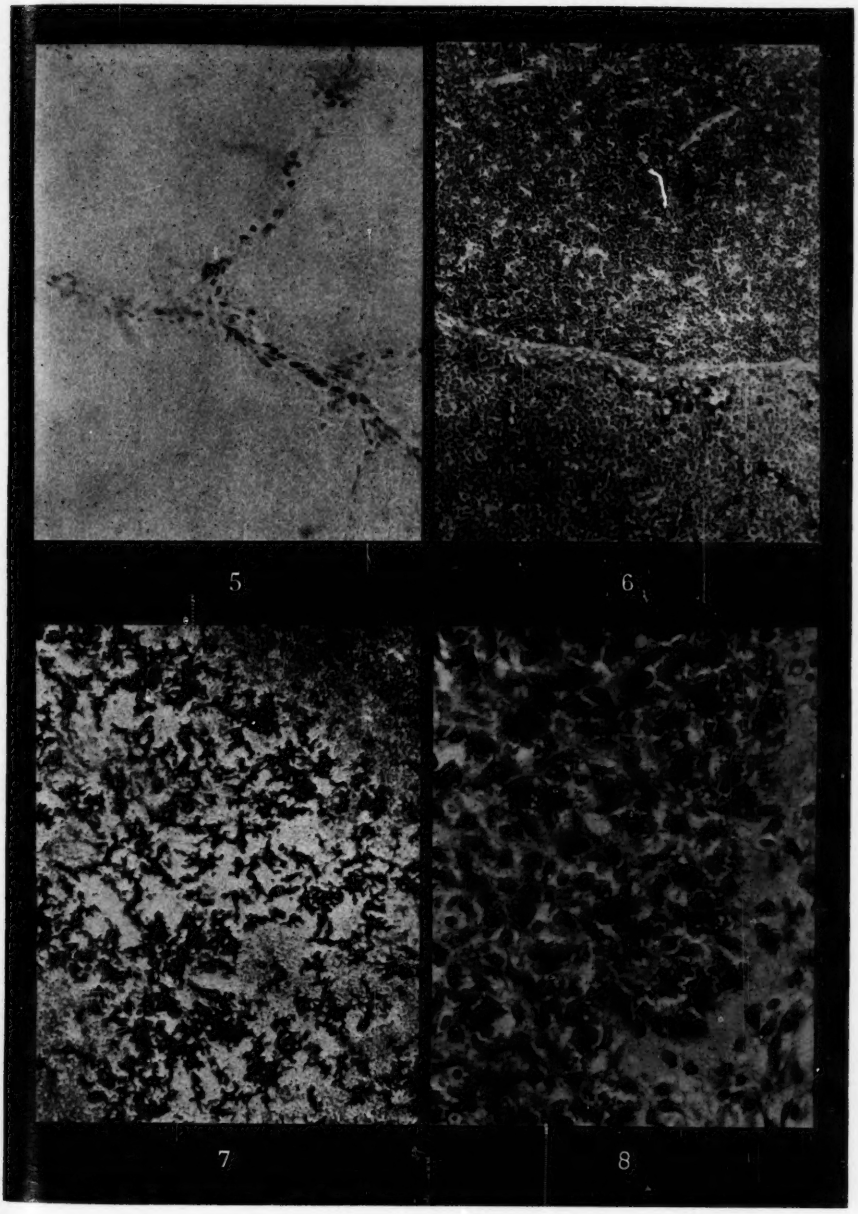

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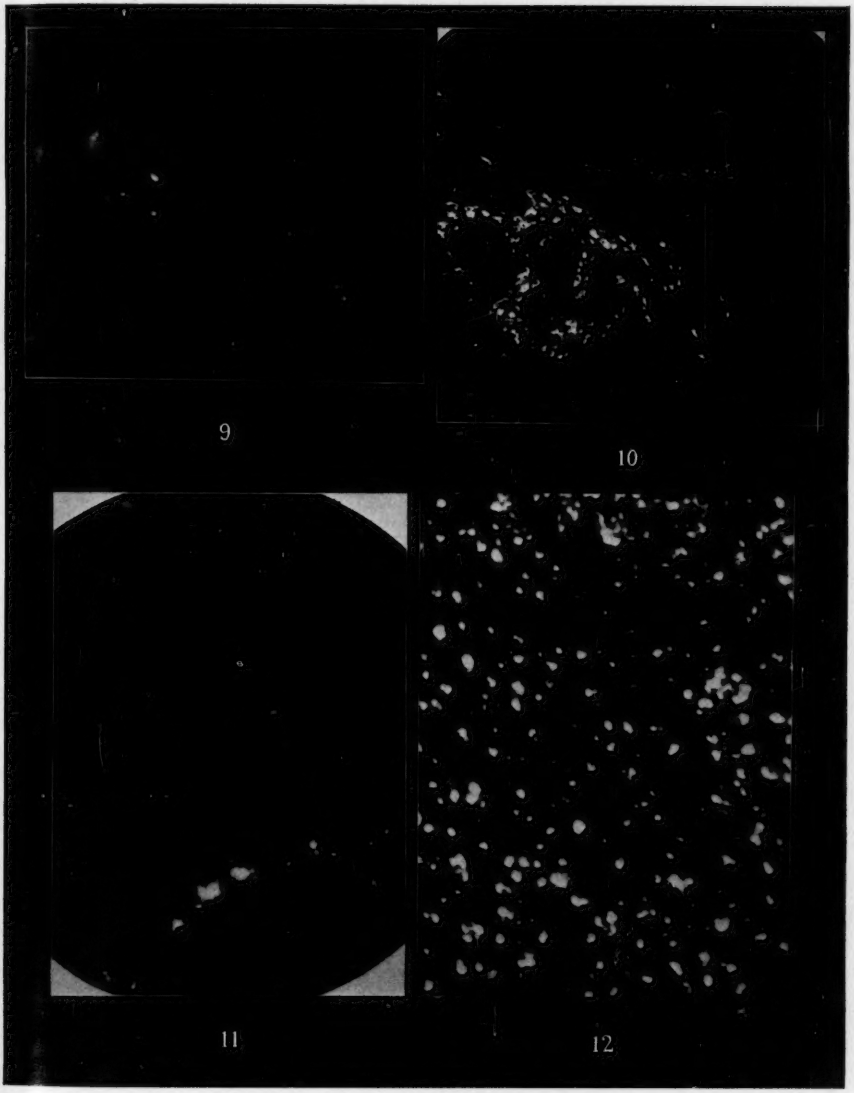

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Marshall and White.

of the incised area (Fig. 8). With metallic impregnation methods a large number of metalophil cells were seen in the same area, many showing transitional forms resembling those seen in the microglia of the brain following cerebral injury.

Distribution of Pneumococcal Polysaccharide in the Normal and Injured Thymus Following Intravenous Injection

Seven guinea-pigs and 2 rats were employed. In the normal and injured thymus the distribution of injected polysaccharide closely followed that of vital dyes. Intense specific fluorescence was observed in cells in the capsule and interlobular connective tissue of the gland (Fig. 9). Marked fluorescence was also seen in the sinus-lining cells of the small lymph nodes attached to the thymic surface in the rat (Fig. 10) and in lymph nodes from other areas of the body. In animals killed 48 hr. after injection polysaccharide was present in considerable amounts in the lumen of blood vessels (Fig. 11). Some non-specific fluorescence was seen in the Hassall bodies (Fig. 11) and in the granules of eosinophil and neutrophil leucocytes in the medulla of the thymus. Only very slight specific fluorescence was observed in cells in the normal thymic cortex or medulla mainly in the region of small blood vessels, a result similar to that observed in the mouse thymus by Coons, Leduc, and Kaplan (1951) employing protein antigens. Following cautery injury in the guinea-pig large numbers of polysaccharide-containing fluorescent cells were observed in thymic tissue adjacent to the injured area (Fig. 12). Hassall bodies in the region of injury formed cyst-like structures containing cells with included fluorescent material.

DISCUSSION

It has been shown that direct injection of antigens into the thymus produces the usual histological changes associated with antibody formation in other lymphoid organs in contrast to the complete lack of cellular reactivity observed by others following introduction of antigens into the blood stream. This failure of reaction to circulating antigen may be attributed either to a barrier against the entry of antigens into the thymus or to the lack of a suitable phagocytic mechanism to segregate it in the gland. The presence of large numbers of reticulo-endothelial cells, as shown by metallic impregnation methods for microglia, in the normal thymus and the rapid phagocytosis of both vital dye and antigenic material following local injury suggest that a barrier exists between the blood and the parenchyma of the thymus similar to that between the blood stream and the central nervous system ; admission of foreign material in quantity to the thymus occurring only following a change in local vascular permeability. The existence of such a barrier would therefore preclude any cellular reaction of the normal thymus to circulating antigen and any antibody formation in the organ.

These observations provide a basis for a possible interpretation of the nature of the lesion in the human thymus in myasthenia gravis. Between 60–70 per cent of cases of myasthenia (Castleman, 1955), show the presence of germinal centres in the medulla of the thymus, structures histologically identical with those seen in lymph nodes following repeated antigenic stimulation. The interpretation of this finding in the human thymus has been a matter of difficulty however, both owing to uncertainty about the significance of formation of germinal centres in other lymphoid tissue and also to the lack of production of a similar lesion in the

the thymus by experimental methods. There is now evidence however that production of germinal centres in lymph nodes may represent a reaction to antigenic stimuli which under certain circumstances results in demonstrable production of specific antibody or γ-globulin (White 1960*b*; Ortega and Mellors 1957), and it has been shown here that the thymus may also form germinal centres *ab initio* as a response to an antigen.

These observations suggest that the thymic lesion in such cases of myasthenia gravis may represent an immune response to an antigen. If the human thymus possesses a barrier to circulating antigen similar to that in animals such an immune response must arise primarily in the thymus gland. This hypothesis would indicate that the thymus is the primary agent in myasthenia gravis and not that a common disturbance gives rise both to the myasthenia and to the changes in the gland as suggested by Havard and Bodley Scott (1960), a conclusion which is also difficult to accept in view of the absence of reaction in other lymphoid organs in myasthenics (Castleman, 1955). It would further indicate that the basic disturbance in myasthenia gravis is immunological in nature, and is not related to the secretion of a hormonal agent by the gland. This view is compatible with both the presence of the characteristic " lymphorrhages " in the muscles of myasthenics a lesion which is also seen only in the muscles in rheumatoid arthritis; and with the predominantly female sex distribution, as often observed in auto-immune disease, of patients showing germinal centre formation in the thymus. Strauss *et al.* (1960) have recently claimed that the serum of patients with myasthenia gravis contains an antibody-like entity which reacts with homologous skeletal muscle and Simpson (1960) has suggested on clinical evidence that myasthenia gravis is an auto-immune disorder in which the thymus reacts to protein derived from the motor end plates. While our findings agree with the hypothesis of an auto-immune basis for this disease they are not compatible with the concept that the thymus reacts to circulating end plate protein or other muscle tissue antigen. On the other hand, the histological appearances in the myasthenic thymus may be compared with the histological reactions at the local injection site in the thymus of a small dose of a bacillary vaccine (TAB), or with those seen in the thyroid in Hashimoto's disease, which also appear to represent the response to a locally escaping antigen (White, 1957) and which prominently feature lymphoid nodules with germinal centres as a part of this immune cellular reaction. If the thymus gland is the primary source of the disease then either a common antigen must be shared by the thymus and motor nerve endings or according to the concepts of Burnet (1959), proliferation of a " forbidden clone " capable of forming antibody or reacting immunologically against nerve endings or muscle components must arise spontaneously in the gland. If the thymus is shielded by a blood-thymic barrier from contact with circulating proteins, it would appear a particularly favourable site for such clonal growth to take place, as contact with antigen is a mechanism postulated by Burnet for the destruction of such " forbidden clones " as are capable of immune responses against an individual's own tissues.

Further investigation is required of the finding of the presence of specific antibody, as reported here, and of gamma globulin (Gitlin, Landing and Whipple, 1953) in some Hassall's corpuscles. It is tempting to assume a common origin and function between the entodermal cells of such structures and the entodermal epithelial cells, which, according to Ackerman and Knouff (1959) and Burnet (1961) give rise to the cells of the germinal centres in the lymphoid nodules of the

bursa of Fabricius in the chicken. The latter shares with the thymus an epithelial origin from the gut and appears to resemble it ontogenetically by progress to a peak size at 4 months in the chicken and then by slow atrophy during the remainder of the bird's life.

SUMMARY

It has been shown that direct injection of antigens into the thymus of guinea-pigs induces the formation of germinal centres and plasma cells and the production of antibody.

The normal rat and guinea-pig thymus possesses a barrier against the entry of vital dyes or antigens from the blood stream into the gland which may be broken down by local trauma. The significance of these findings is discussed, especially in relation to the pathogenesis of myasthenia gravis.

We are indebted to Professor I. Doniach for helpful criticism of this paper and to Dr. J. H. Humphrey for the sample of pneumococcal polysaccharide employed.

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SOME ASPECTS OF COMPENSATORY HYPERPLASIA OF THE KIDNEY

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Received for publication March 10, 1961

THE mechanisms controlling tissue growth and regeneration are largely unknown, although disturbances of these may well play an important part in many diseases. One of the basic problems in any study of these processes is to estimate the rate at which new cells are formed under different conditions. In organs with only diploid cells, the rate of incorporation of radioactive materials into DNA will give a general index, but when the organ contains cells of several histological types, the most informative method is still mitotic counts. A fair amount is known about the later stages of the enlargement of the remaining kidney after unilateral nephrectomy, but the early changes and the changes in the mitotic rate of various cell types have not been examined in any detail. As the kidney may have certain advantages as an experimental model for the study of regeneration, the normal mitotic rate in the kidney and the changes during hyperplasia have been examined with special reference to the first 3 days after unilateral nephrectomy. At the same time, changes in the basement membranes and in stainable fat were sought, as these are early and prominent in the regenerating liver (Aterman, 1952; Stowell, 1948).

METHODS

Male albino rats of an inbred strain of Wistar origin maintained on a diet of MRC rat cubes and weighing 112–180 g. were used. Under ether anaesthesia and with aseptic precautions the right kidney was exposed through a short incision in the loin. The renal pedicle was ligated, sparing the adrenal, and the kidney removed: the incision was then closed in 2 layers with cotton sutures. Sham operations in which the kidney was exposed, handled but not removed were performed in other animals. Most operations were performed at 2–4 p.m., and groups of animals were killed after 8, 16, 24, 32, 40, 48, 56, 64 and 72 hr. and after 4, 6, 8 12, 16 and 20 days. To investigate diurnal variations, other groups of animals were operated upon at 10–12 p.m., and at 6–8 a.m. The remaining kidney was removed immediately after death, bisected transversely at the level of the hilum, and fixed in mercuric chloride-formalin and in 10 per cent formalin: transverse paraffin and frozen sections were prepared from the level of the hilum. Frozen sections were stained with Sudan IV, and paraffin sections with haematoxylin-eosin and with PAS-haematoxylin.

For counting mitoses, the kidneys were zoned in the manner described by McFarlane (1941). Peter's sub-division (Peter, 1908) is much less convenient in the rat. By McFarlane's method the kidney is divided into (1) the outer cortical zone, containing most of the glomeruli and the convoluted portions of the proximal and distal tubules, (2) the inner cortical zone, merging into the former, containing few glomeruli, and being mainly composed of the straight portions of the proximal tubules, the ascending broad limbs of the loops of Henle, and collecting tubules, (3) the outer medullary zone, containing mainly the ascending limbs, thin loops and collecting ducts, and (4) the inner medullary zone containing the narrow limbs of the loops of some nephrons, and the larger collecting ducts only. These zones were represented to a varying extent in the sections which were rarely exactly median. To allow for this, a

small area of the high power field, measuring 111 × 67 μ, was delineated by an eyepiece graticule and the number of mitoses of each cell type counted in 150 of these areas in each zone. If more than one section was needed, they were spaced at least 30 μ apart, to avoid counting the same mitosis twice. Prophases before the dissolution of the nuclear membrane, and late telophases were excluded.

RESULTS

In otherwise normal rat kidneys lymphocytic infiltration is common, and spontaneous hydronephrosis is occasionally found : any kidney showing hydronephrosis or more than a very occasional small group of lymphocytes microscopically was discarded, and the experiment repeated.

Nearly all the mitoses in the normal kidney and during hyperplasia occur in the proximal tubules, the collecting tubules, the ascending limbs or the distal tubules. The latter 2 respond differently during hyperplasia and have therefore been classed separately. No significant difference could be found between the right and left kidneys of the 18 sham operated animals, and in view of this, the results of some other experiments which were identical except that the left kidney rather than the right was removed have been included.

Normal mitotic rate and diurnal variation

Mitoses were counted in 3 groups of 40 kidneys removed at 6–8 a.m., 2–4 p.m., and 10–12 p.m. When the results were plotted as frequency histograms, it was obvious that the distributions were not normal, most being strongly positively skewed ; Fig. 1 shows the histograms for the total numbers of mitoses of the 4 main cell types in kidneys removed at 2–4 p.m. This means that the standard deviations will not give reliable frequency limits and to show changes in the mitotic rate, the best indices for the control of population would seem to be the mean and some estimate of the upper limit. In Table I the means and the values below which 95 per cent of all normal readings fall are given for the total counts, and for the counts for each zone and cell type.

In 18 mitoses, just under 3 per cent of the total, the cell type could not be determined. Mitoses were very rarely seen outside the renal tubules : only 4 connective tissue mitoses were seen in the 40 kidneys removed at 2–4 p.m., 3 in those removed at 10–12 p.m., and 2 at 6–8 a.m. Three other mitoses were seen, 2 in thin loops, and 1 in the visceral epithelium of a glomerulus.

There is an obvious and uniform diurnal variation, the counts at 2–4 p.m. being approximately double those at 6–8 a.m., when the rate was lowest for almost all the classes shown in Table I.

TABLE I

	2–4 p.m.		10–12 p.m.		6–8 a.m.	
	Mean	95 per cent upper limit	Mean	95 per cent upper limit	Mean	95 per cent upper limit
Total counts	7·6	18	4·2	9	3·6	8
Outer cortex	2·9	8	1·7	6	1·1	4
Inner cortex	2·1	6	1·1	4	1·0	4
Outer medulla	2·6	8	1·3	5	1·5	5
Inner medulla	0·1	2	0·02	1	0·0	1
Proximal tubules	4·0	10	2·1	7	1·6	6
Distal tubules	0·6	3	0·4	3	0·3	3
Ascending limbs	2·2	7	1·1	4	1·3	4
Collecting ducts	0·4	3	0·3	3	0·2	2

Compensatory hyperplasia

The mitotic counts during hyperplasia in the animals operated upon at 2–4 p.m. are given for the 4 predominant cell types in Figs. 2 and 3. The dotted lines show the levels below which 95 per cent of the counts in normal animals fall, the levels usually being different at different times of day. The means for the various groups are joined by the continuous line. The results for the different zones are apparently

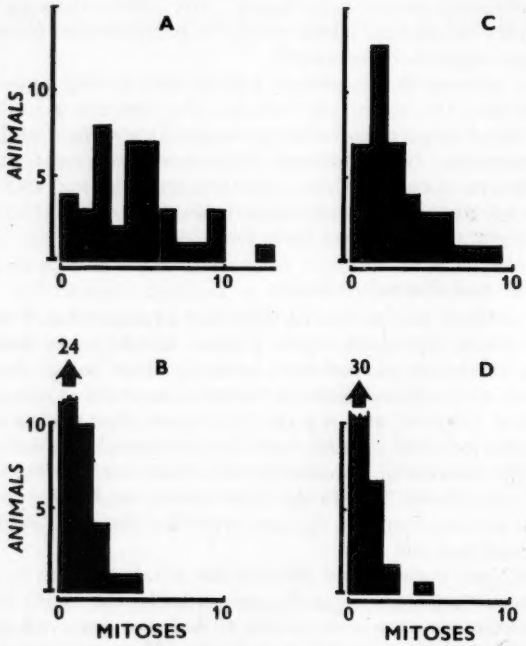

FIG. 1.—Frequency histograms for mitotic counts in kidneys removed at 2–4 p.m. Ordinates: number of animals; abscissae: mitotic counts. (A) Proximal tubules, (B) Distal tubules, (C) Ascending limbs, (D) Collecting ducts.

largely determined by the predominant cell type and so are not given in detail. The means for the different zones are shown in Fig. 4.

The sham-operated animals show no significant increase in mitotic activity at any time. In the operated animals the proximal tubules show some increase in mitotic activity at 24 hr. followed by a rapid climb to a maximum at 40 hr. and an equally sharp fall to normal levels by 56 hr.; there is a second smaller rise at 3 days continuing until at least 12 days. The distal tubules have a single, less well marked increase in activity at 32–40 hr.: the ascending limbs have an increased rate at 40 hr., but the largest rise occurs in a second later peak at 3 days. The collecting ducts show a similar but less well marked pattern with peaks at 40 hr. and 4 days. As in the normal kidney, mitoses were almost confined to the tubules. Two mitoses were seen in the visceral epithelium of glomeruli; a single connective tissue

mitosis was seen in 4 of the 12 kidneys removed at 48 hr. after operation, and in one of the 15 removed after 24 hr.: there were three in one of the 8 removed at 3 days. These counts are too low to more than suggest a possible connective tissue response around 48 hr. The cell type of 17 mitoses (1·3 per cent of the total) could not be decided.

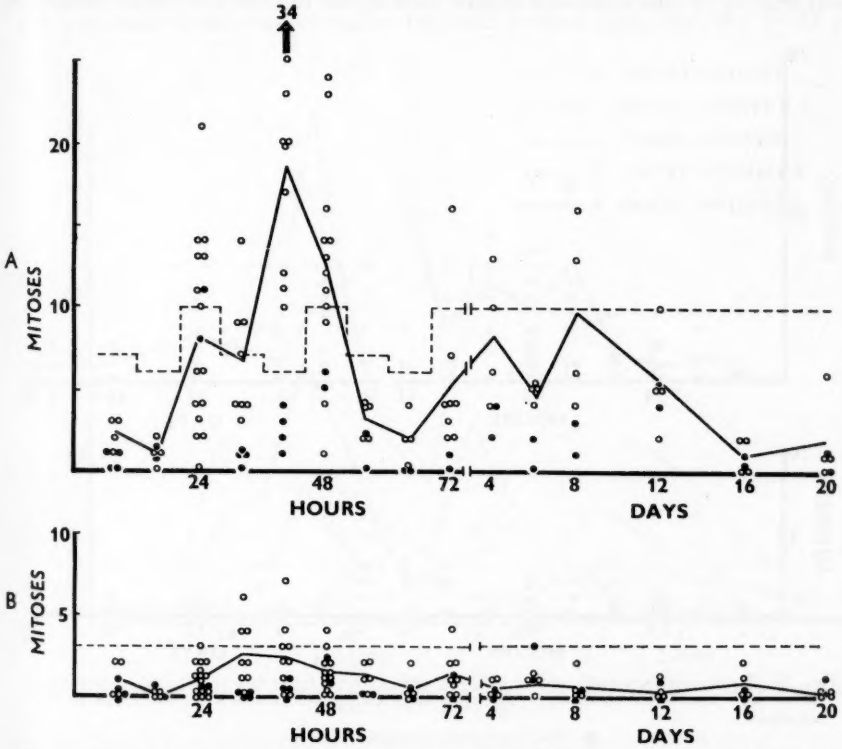

FIG. 2.—Total mitotic counts of the different cell types. Ordinates: number of mitoses; abscissae: time after operation. The time scale differs before and after the break in the abscissae.

● Sham operated animals.
○ Unilaterally nephrectomized animals.
(A) Proximal tubules, (B) Distal tubules.

The outer cortex showed the greatest mitotic activity, whereas only 10 mitoses were seen in the inner medulla throughout the whole course of hypertrophy. The pattern of the mitotic response, though not the absolute level seemed to be determined by the predominant cell type. Although both the collecting ducts and the the ascending limbs lay largely in the outer medulla, and showed a similar biphasic response, in the inner cortex the proximal tubules had the characteristic single peak at 40 hr., whereas the ascending limbs in the same zone showed a biphasic response with a maximum at three days.

Mitotic counts were made per unit area rather than per 1000 cells. To find the approximate rate as a proportion of cells present the number of cells of each type were counted in every 10th field of the 150 fields examined in each zone, in 15 control animals and in 2 from each of the post-operative groups. There was no significant variation with time of day or with time after operation. The average total number of cells examined in each section was 16, 290 (s.d. 1029), comprising

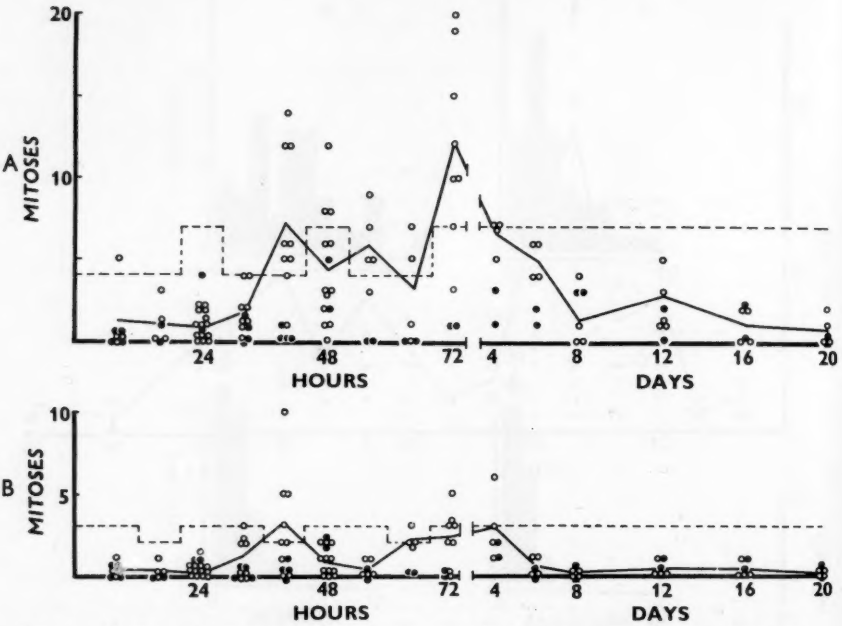

FIG. 3.—Total mitotic counts of the different cell types. Ordinates: number of mitoses; abscissae: time after operation. The time scale differs before and after the break in the abscissae.

● Sham operated animals.
○ Unilaterally nephrectomized animals.
(A) Ascending limbs, (B) Collecting ducts.

6400 proximal tubule, 1270 distal tubule, 4660 ascending limb and 3960 collecting duct cells (1290 of the latter if the mitotically inactive inner medullary zone containing the thin loops and the largest collecting ducts only is excluded). From these figures the approximate rate per 1000 cells can be calculated. and the results for the 4 main cell types are shown in Fig. 5. The highest rate in unoperated animals was 0·61/1000 in the proximal tubule cells at 2–4 p.m., and the highest rate during hyperplasia, 4·38/1000, was reached in the proximal tubule cells of the outer cortex at 40 hr. It can be seen from Fig. 5, that if the inner medulla is excluded, the absolute mitotic rates of the various cell types reach comparable levels, in contrast to the considerable differences observed for the mitotic counts per unit area.

Effect of the diurnal rhythm

The shape of the mitotic response differs when operations are carried out at different times of day. Similar experiments were performed (over the first 48 hr.) starting at 10–12 p.m. or 6–8 a.m. The average total counts, with the results of the experiment starting at 2–4 p.m. for comparison, are shown in Fig. 6 with the number of animals in each group in brackets over the appropriate point. The 6–8 a.m. experiment has an earlier less well marked peak than the 10–12 p.m.

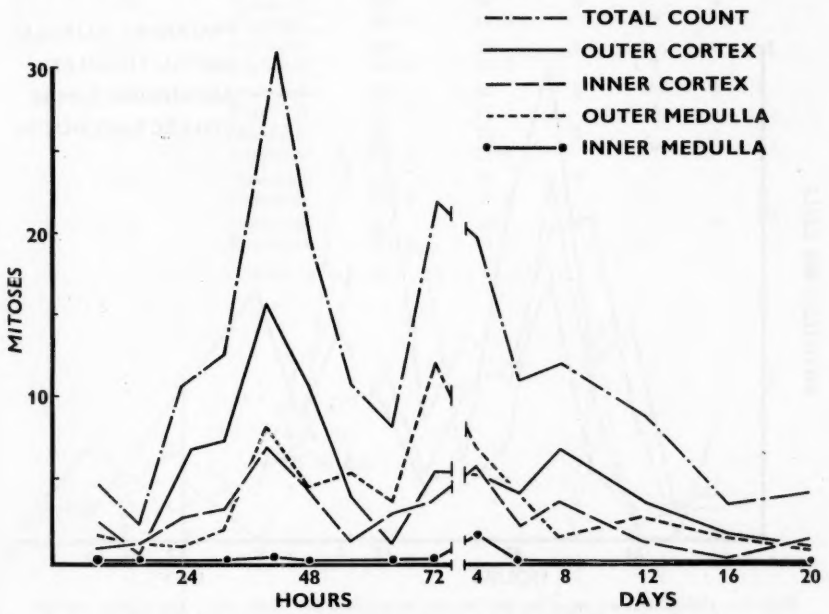

FIG. 4.—Average mitotic counts per standard area against time for the 4 zones separately and for all zones together.

experiment, which has a significant rise only at 40 hr.; the 2–4 p.m. results have an intermediate form. These differences are too great to be accounted for by chance sampling errors. When the variances of the groups are compared by Snedecor's F, and the means by the " t " test where appropriate, there is a significant difference ($P = < 0{\cdot}05$) between at least 2 of the group means at all times from 24–48 hr., and at 32 hr. the chance probability of the differences is less than 0·01 in each case.

Distribution of mitoses

During counting, mitoses often appeared to occur closer together than would be expected purely by chance. To examine the distribution, several sections, spaced more than 30μ apart, were taken from 8 kidneys with high mitotic counts, and 600 fields counted in the outer cortical zones of each. Nearly all the mitoses

occurred in the proximal tubules, and these and the distal tubule mitoses were examined separately. The number of fields containing 0, 1, and 2 or more mitoses was determined, and compared with the number expected in each of these classes from a purely random (Poissonian) distribution. The results for the proximal tubules are shown in Table II. As the "expected" value in the 2 or more class was often very low, the results in several animals have had to be pooled to apply significance tests, but it is obvious that clumping is not very marked if in fact it exists: this is confirmed by a χ^2 test which gives a chance probability of these

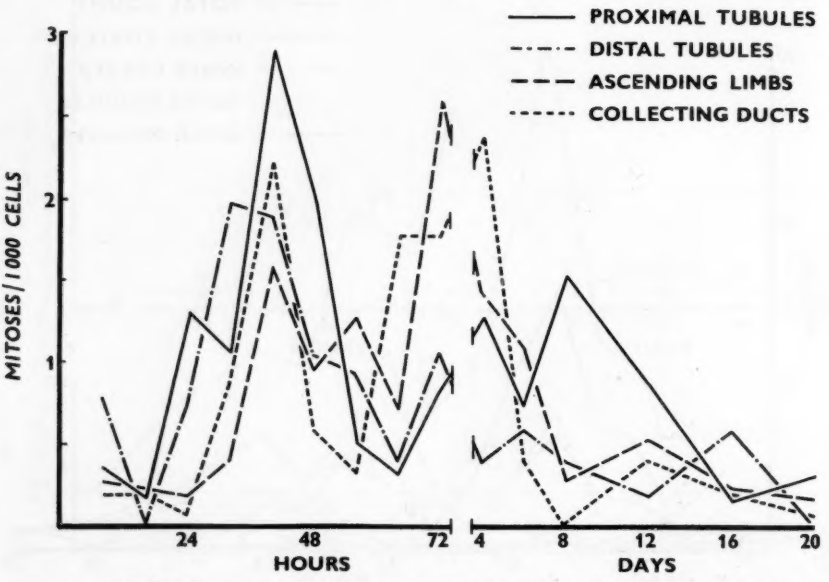

FIG. 5.—Calculated average mitotic counts as mitoses per 1000 cells: the figures for the collecting duct cells exclude the cells of the inner medulla.

results occurring with a purely random distribution of $> 0{\cdot}1$. The distal tubular counts were unhelpful, as more than one mitosis was seen only once in the 4800 fields examined.

Basement membrane changes

Paraffin sections (5 μ) of kidneys removed at operation and of those removed at the end of the experiment were stained by the P.A.S. method and lightly counterstained with haematoxylin. The basement membranes of the tubules and glomeruli stood out clearly, but no change could be detected at any interval after nephrectomy.

Changes in stainable fat.

Frozen sections of the kidneys were stained with Sudan IV. No significant amounts of fat were seen in the cortex or outer medulla before or after operation.

TABLE II

		Number of mitoses/field				
		0	1	2	3	>3
A 552 .	Observed	556	42	2	0	0
	Expected	555·8	42·6		1·6	
A 532 .	Observed	524	72	4	0	0
	Expected	525·2	70·0		4·8	
A 542 .	Observed	569	26	5	0	0
	Expected	565·0	33·9		1·2	
A 562 .	Observed	559	40	1	0	0
	Expected	559·5	39·2		1·3	
A 312 .	Observed	532	59	9	0	0
	Expected	527·9	67·7		4·4	
A 362 .	Observed	521	72	6	1	0
	Expected	519·0	75·3		5·7	
A 332 .	Observed	518	73	7	1	1
	Expected	512·9	80·4		6·7	
A 822 .	Observed	521	70	8	1	0
	Expected	517·4	76·7		5·9	

Total $\chi^2 = 6{\cdot}83$. 4 d.f. $p > 0{\cdot}1$.

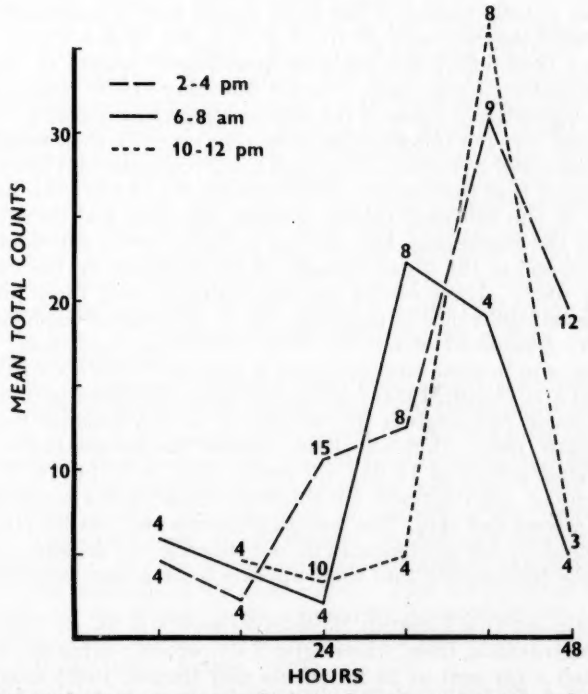

FIG. 6.—Mean total mitotic counts over the first 2 days in experiment started at 2–4 p.m., 10–12 p.m., or 6–8 a.m.

In the inner medulla, tiny sudanophilic droplets could be seen. These did not appear to lie within the cytoplasm of the cells of the collecting ducts or of the thin loops, but lay within the stellate connective tissue cells between these. The droplets ranged from the barely visible up to about 2 μ and were usually most easily visible around the nucleus. This distribution agrees with that previously given by Rice and Jackson (1934), although they noticed occasional droplets also in cortical tubules and in the epithelium of the thin loops. There was no detectable change in the size, number, or distribution of the droplets at any time after operation.

DISCUSSION

Assessment of changes in mitotic rate is difficult as the range of normal mitotic activity is fairly wide, and occasional inexplicably high values are found. The frequency distribution is often strongly positively skewed. This is unfortunate, as this means that to use the standard deviation to establish chance probabilities assuming a normal distribution as is customarily done could give misleading results with small samples, and makes any simple mathematical estimate of significance difficult : control groups have to be large before the importance of a variation in the mean can be assessed. The diurnal variation is marked ; and the results are in general agreement with those of Blumenfeld (1943), who also found a diurnal rhythm in the mitotic counts of the renal cortex with a maximum at 2–4 p.m., though he placed the minimum at 10–12 p.m. rather than 6–8 a.m.

There have been only a few previous quantitative studies of the mitotic rate during compensatory hyperplasia. Carnot and May (1938) counted the number of mitoses in the different zones of the regenerating kidney after giving colchicine, but as they used very few rats at rather large time intervals their results are difficult to assess. Dustin and Zylbersac (1939) also using colchicine did not give numerical results, but stated that there was a marked response at 24 hr., the mitotic response being mostly in the proximal tubules between the first and fourth days, on the fourth day in the glomerulus, and on the seventh day in the thick limbs of the loops of Henle and in the distal tubules : none were seen in the thin loops or in the collecting ducts. These results are very different from those presented here ; the only apparent difference in technique lies in their use of colchicine. Although this is usually considered merely to block mitoses in metaphase, this is by no means certain, and in some circumstances it appears to have a stimulating action as well (Eigsti and Dustin, 1955) : certainly, when compared with normal mitotic counts in tubular regeneration in the kidney, it can produce very anomalous results (Bartman, 1960). Rollason (1949) counted the mitoses in the various zones of the remaining kidney of 14 day old rats 1, 2, 3, 6, 8, 14, 16, 22 and 56 days after nephrectomy with appropriate controls. There was a significant rise in all zones on the second day only. The reports of Ogawa and Sinclair (1958) and Frank (1960) are based on few animals and do not describe the responses of the various cell types ; the former note that there appears to be a later response in the outer medulla.

The mitotic activity found in these experiments is by no means as brisk as that of the regenerating liver, where after a 68 per cent hepatectomy the mitotic rate may reach 4 per cent at 24 hr. (Brues and Marble, 1937), compared with at most 0·43 per cent in the kidney (after some 50 per cent of the renal tissue has been removed). However, though small in absolute terms, the rise is still well

marked ; at 40 hr. the mitotic rate in the proximal tubules has a mean value 11·5 times the normal mean, and three times the 95 per cent normal upper limit. Individual values were very variable, but all were above the normal range.

The most striking thing about the responses of the various cell types is the rapidity with which the mitotic rates change during the first few days. The peaks can easily be missed ; 8 hr. before and 8 hr. after the 40 hr. peak in the proximal tubules the rate was 30–60 per cent lower, and individual values at these times frequently fell within the normal range. The controlling mechanisms are unknown, though for the regenerating liver attempts have been made to fit the pattern of response to mathematical relationships implying that the rate at which new cells are formed is inversely proportional to the tissue deficit (Brues and Marble, 1937) : even if this is true, it is complicated by the marked diurnal variation in the mitotic rate since discovered in the regenerating liver (Jaffe, 1954). Certainly the rapid fall in the mitotic rate of the proximal tubule cells from the 40 hr. peak to effectively normal levels at 64 hr. is entirely disproportionate to changes in the deficit of renal tissue during this period. The total number of new cells formed in excess of normal growth can be calculated if it is assumed that the normal duration of a mitosis is about 40 min.—a reasonable assumption in mammals—and that the mitotic rate is constant from 4 hr. before until 4 hr. after each experimental point. By 68 hr., the number of excess proximal tubule cells formed on these assumptions is only 4·3 per cent, which is only a small fraction of the 40–70 per cent increase expected eventually from the weight gains of the kidney during compensatory hyperplasia (Addis and Lew, 1940 ; MacKay, Addis and MacKay, 1938) and this low figure is supported by the finding that most of the increase in the DNA content of the regenerating kidney does not start until several days after operation (Mandel, Mandel and Jacob, 1950 ; Miyada and Kurnick, 1960). This suggests that the changes in mitotic rate must be determined by some other mechanism than a simple function of the tissue deficit. The mitotic rates found during the later part of the experiment can only be regarded as approximate. The kidney increases in weight until at least 20 days, and there is probably a continuing formation of new cells during the whole period. However, the increases in rate need only be very small. If a mitosis takes about 40 min. to complete, a 70 per cent hyperplasia could be produced in this time by a steady increase in rate of only 1 mitosis/1000 cells, an increase which would require a very large number of animals to define accurately.

The patterns of response obtained when experiments are started at different times of day differ quite markedly, though less than the extreme variations found by Jaffe (1954) in the liver during regeneration. It was not possible to explain the differences between these patterns on various assumptions about the nature of the diurnal changes. These differences, as well as the difference in the control figures at different times of day, are important in comparing the results of different experiments. The exact form of the diurnal rhythm and mitotic response may well be strain-specific, and like the relation between kidney weight and body weight would need to be determined for each colony. This is supported by the slightly different results for the diurnal rhythm found by Blumenfeld (1943) and the more marked later response during hyperplasia noted incidentally by other workers (Robertis and Poch, 1947 ; Sulkin, 1949).

In the liver, the sinusoidal basement membrane shows a most interesting series of changes during regeneration starting as early as 3–6 hr., and it has been

suggested that these bear some functional relationship to the onset of mitosis (Aterman, 1952): however, as the basement membranes of the renal glomeruli and tubules are not entirely analogous to those of the hepatic sinusoids, no conclusions can be drawn from the absence of similar changes in the kidney.

The absence of changes in the stainable fat also differs from the marked early accumulation in the regenerating liver (Stowell, 1948): the functional significance of this accumulation is unknown, but the present results suggest that it is more likely to be a reflection of the special relationship of the liver to fat metabolism than a general characteristic of regenerating tissues.

SUMMARY

A survey was made of the mitotic activity of the various cell types and zones in the normal kidney of the albino rat, and in the kidney undergoing compensatory hyperplasia. There is a well-marked diurnal rhythm in the normal kidney. During hyperplasia there was a sharp peak of mitotic activity in the proximal tubules 40 hr. after operation with smaller increases also maximum at that time in the distal tubules. The ascending limbs and collecting ducts showed 2 peaked responses with maxima at 40 hr. and at 3 and 4 days respectively. No definitely significant increase in the number of connective tissue mitoses was found at any time. The mitotic activity was greatest in the outer cortex; mitoses were extremely rare in the inner medulla. No changes could be found in the basement membranes of the tubules or in the distribution of stainable fat.

I am most grateful to Professor A. C. P. Campbell for his interest and encouragement, and to Dr. R. Ollerenshaw, of the Department of Medical Illustration, Manchester Royal Infirmary, for preparing the diagrams.

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THE RELATION BETWEEN THE FIXATION AND INACTIVATION OF TETANUS TOXIN BY GANGLIOSIDE

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Received for publication March 14, 1961

IT has been shown that ganglioside acts as a receptor for tetanus toxin in nervous tissue: studies with a crude ganglioside-cerebroside complex showed that the fixation of tetanus toxin appeared to be specific since no other toxin or protein tested was fixed at physiological salt concentration, except, to a lesser extent, tetanus toxoid (Van Heyningen, 1959; Van Heyningen and Miller, 1961). Van Heyningen (1959) also showed that tetanus toxin was inactivated when stood with ganglioside for some time. This inactivation increased with time and temperature and was diminished in the presence of a protective colloid such as gelatin (Van Heyningen, unpublished). North and Doery (1961) have confirmed the inactivation of tetanus toxin by ganglioside, and have shown that other toxins (*viz.* diphtheria and staphylococcal toxins) are also inactivated. They suggest that this finding is " contrary to Van Heyningen's conception [that ganglioside acts as a specific receptor for tetanus toxin] ". However, since the crude tetanus toxin receptor has been shown not to fix diphtheria toxin (Van Heyningen, 1959), it does not seem likely that inactivation and fixation of toxin are aspects of the same phenomenon. Moreover, tetanus toxin fixed by crude receptor (Van Heyningen, 1959) or by purified ganglioside (Van Heyningen, unpublished), is not necessarily inactivated. In this paper the failure of ganglioside to fix diphtheria toxin is demonstrated.

METHODS

Studies of toxin-ganglioside interaction were made in the Spinco Model E analytical ultracentrifuge by the method of Van Heyningen and Miller (1961). The tetanus toxin was a 75 per cent pure preparation kindly provided by Dr. C. G. Pope and Dr. R. O. Thomson of the Wellcome Research Laboratories (Van Heyningen, 1959); the diphtheria toxin was a highly purified 5-times crystallized " diphtheria toxin protein " provided by Dr. C. G. Pope; ganglioside was prepared from beef brain and purified by the method of Van Heyningen and Miller (1961). All reactants were dissolved in 0·1 M-phosphate buffer pH 7.

EXPERIMENTAL

The figure shows the schlieren diagrams obtained after centrifuging solutions containing toxin alone, or toxin and ganglioside, for 1 hr. at 42,040 r.p.m. Diagram " *a* " is of 5 mg. tetanus toxin alone/ml.; " *b* " is the diagram of a mixture of 5 mg. tetanus toxin and 2·5 mg. ganglioside/ml. The ganglioside has fixed toxin and reduced the area of the toxin peak (in the original negative) from 32·4 to 7·7 sq. mm., *i.e.* 3·8 mg. of toxin have been fixed by 2·5 mg. of ganglio-

side. (In fact this amount of ganglioside is excessive, and has fixed all the available toxin—the remaining unfixed protein is the 25 per cent impurity in the toxin preparation [see Van Heyningen and Miller, 1961]). Diagram "*c*" is of 5 mg. diphtheria toxin protein alone/ml.; "*d*" is the diagram of a mixture of 5 mg. diphtheria toxin and 5 mg. ganglioside/ml. There has been no diminution in the area of the diphtheria toxin peak (20·8 sq. mm. in "*c*"; 21·0 sq. mm. in "*d*"), despite the fact that twice as much ganglioside was used as fixes at least 3·8 mg. tetanus toxin.

DISCUSSION

It is clear that ganglioside does not fix diphtheria toxin although, according to North and Doery (1961), it may inactivate it under suitable conditions. The fixation of tetanus toxin and the inactivation of tetanus and other toxins by ganglioside appear to be unrelated phenomena; the fixation is highly specific, the inactivation is non-specific. What the non-specific inactivation is due to is not clear. Aqueous solutions of ganglioside tend to froth and it was thought that ganglioside might denature proteins by a detergent action, but investigations kindly carried out by Dr. G. Miller of Nottingham University showed that the surface tension of a 1 per cent solution of ganglioside in water (68 dynes/cm.) is very little different from that of water alone (72·8 dynes/cm. at 20°).

I am grateful to Dr. C. G. Pope for providing the tetanus and diphtheria toxin preparations, to Dr. G. Miller for carrying out the measurements of surface tension, to the Wellcome Trustees for the Spinco analytical ultracentrifuge, and to the Office of Naval Research of the United States Department of the Navy for a grant for expenses (Project No. 103–474).

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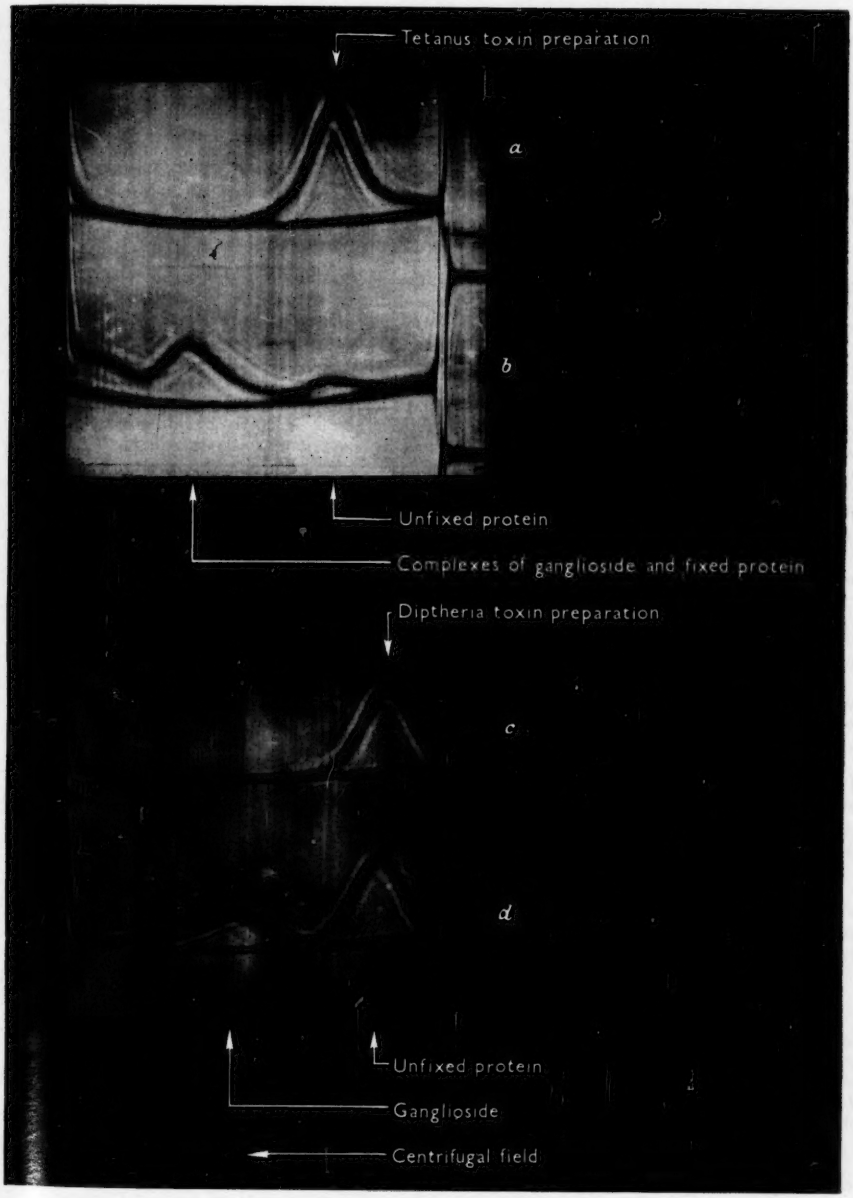
EXPLANATION OF PLATE.

Fig.—Schlieren diagrams observed after centrifuging toxin alone or toxin + ganglioside for 1 hr. at 42,040 r.p.m., phase plate angle 60°.

(*a*) 5 mg. tetanus toxin preparation/ml.;
(*b*) 5 mg. tetanus toxin preparation + 2·5 mg. ganglioside/ml.;
(*c*) 5 mg. diphtheria toxin preparation/ml.;
(*d*) 5 mg. diphtheria toxin preparation + 5 mg. ganglioside/ml.



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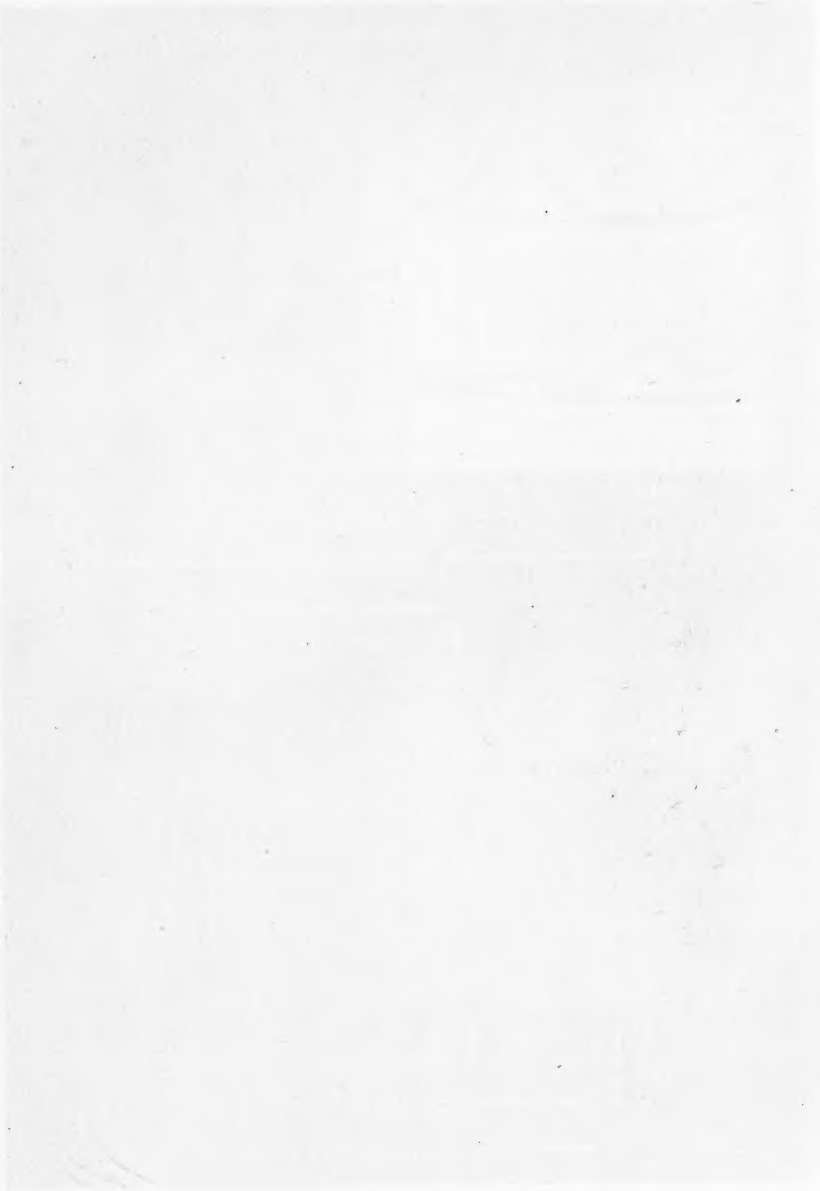

SURVEY OF PAPERS

MAUREEN DALE has reported further work on the effects of the components of adjuvants on the lymphatic vessels in the ears of mice. The constituents were tested singly and in various combinations and the conclusions reached were that the overall effect on the vessels were probably a result of their activity in combination and were not due to any particular constituent (p. 297).

FIELD x-irradiated guinea-pigs with a dose of 150r and 18 hr. later inoculated them intracutaneously with a mixture of human white matter and adjuvant. The skin sensitivity of the animals was assessed after 16 days and again after 28 days. He found that irradiation enhanced the severity of experimental allergic encephalitis and produced no significant diminution of delayed sensitivity (p. 303).

BRIGID BALFOUR, DEBORAH DONIACH, ROITT and COUCHMAN applied sera from cases of Hashimoto's and other diseases of the thyroid to sections of thyroid glands removed from cases of Graves' disease. Such treated sections were then "stained" with a fluorescein conjugated anti-human-globulin rabbit serum. Three sorts of positive reaction were seen; one of which, a uniform fluorescence of the colloid, was characteristic of sera having no precipitating, tanned red-cell agglutinating or complement fixing reaction with thyroglobulin. They demonstrated that this type of fluorescent staining was not due to a blocking antibody to thyroglobulin, but a response to a separate antigen of the thyroid colloid, CA2, and that it was also found in some precipitating sera which had been cleared of precipitin by absorption with thyroglobulin (p. 307).

TAYLOR ROBINSON, ZWARTOUW and WESTWOOD grew the 3 types of poliovirus in suspended ERK cells in nutrient-free saline. By the addition of glucose to the fluid or oxygen to the overlying gas phase in closed vessels, 10 ml. volumes (10^7 cells/ml.) could be made to yield 100 PFU of virus per cell. In larger volumes (up to 1·5 l) suspended cells would yield similarly in the presence of adequate oxygen, but glucose without oxygen would not support maximal virus production (p. 317).

NÁDASDI, pursuing earlier observations of Selye that cardiovascular lesions could be induced by the simultaneous administration of adrenocorticosteroids and inorganic salts, has confirmed that similar lesions are produced by the administration of some salts to rats with unilateral renal ischæmia. The principal features of these two types of cardiac and vascular degeneration have been compared and contrasted (p. 324).

MARY HOLDSWORTH and HODGKINSON have measured changes in hexosamine content of rat kidneys during calcification. When results were expressed on the basis of tissue nitrogen, no departure from normal levels was observed although the calcification may have involved an increased turnover of mucopolysaccharides (p. 331).

JAHIEL and KOFFLER inoculated guinea-pigs with Freund's adjuvant, adjuvant and homologous liver, and liver alone. Animals were killed at intervals up to 60 days after inoculation and the livers examined histologically. They found that animals that received adjuvant alone and adjuvant with liver developed hepatic lesions that were mainly necrotic in the early stages, but later became epithelioid cell granulomata. The addition of liver to the adjuvant increased the number of granulomata, but did not affect the structure of them (p. 338).

GASSNER and WERTHEIMER, studying the connection between carbohydrate utilization and the release of protein by incubated liver and muscle slices, have extended previous work to

tissues of animals with experimental diabetes, thyrotoxicosis, hypothyroidism and carbon tetrachloride poisoning. Factors influencing the release of protein in these pathological conditions have been examined; the electrophoretic behaviour of the components of the protein is discussed (p. 343).

BLACKLOCK and REES have inoculated tubercle bacilli into the adrenals of rabbits and guinea-pigs. They found that lesions were minimal in the cortical zone, but more extensive in the medulla. They consider the slow development of the cortical lesions indicates the action of a hormonal factor (p. 351).

EBBA LUND, LYCKE and SOURANDER have made a cinematographic study of *Toxoplasma gondii* growing in tissue culture cells. They show the parasite dividing by binary fission, rupture of the host cells and rapid invasion of fresh host cells (p. 357).

JACKSON and MORRIS have investigated the effect on growth in serum of *Past. pestis* and certain other bacteria of adding iron. They found that, in the case of *Past. pestis*, growth in serum was greatly enhanced by the addition of ferrous iron. They discuss their results in relation to differences in virulence between strains of *Past. pestis* (p. 363).

GREEN and CHRISTIE gave pregnant rats a single intraperitoneal injection of the pyrrolizidine alkaloid heliotrine. The dose and the day of gestation on which it was given varied. Anomalies were observed in the offspring, especially dwarfism and rib deformities. These were most marked in the young of rats receiving the larger doses around the 12th day (p. 369).

MARSHALL and WHITE injected TAB vaccine or aluminium phosphate precipitated diphtheria toxoid directly into the thymic substance of guinea-pigs and rats. This caused germinal centres to form, with plasma cells and antibody production which they recognized by indirect fluorescent staining. Antigens introduced intravenously did not have these effects in the thymus unless the gland was subjected to local trauma. The authors discuss their results in relation to myasthenia gravis (p. 379).

WILLIAMS investigated compensatory hyperplasia of the kidneys of male albino rats following unilateral nephrectomy. The nephrectomies were carried out at different times during the day and mitotic activity in several zones of the normal control kidneys was compared with that found in the kidneys undergoing compensatory hyperplasia. He found that during hyperplasia there was a sharp peak in the mitotic count in the proximal tubules with a lesser one in the distal tubules 40 hr. after operation. The ascending limbs and collecting ducts also showed mitotic responses maximal at 40 hr. and 3–4 days respectively. The mitoses were most numerous in the outer cortex and were very rare in the inner medulla while a significant increase in the number of connective tissue mitoses could not be detected (p. 386).

VAN HEYNINGEN demonstrated by ultracentrifugation that beef brain ganglioside, which fixed tetanus toxin, did not fix diphtheria toxin. Inactivation of the latter by ganglioside, as found by other workers, was, he says, nonspecific and independent of fixation (p. 397).

ERRATUM

In the paper "Induction of Staphylococcal Infections in Mice with Small Inocula Introduced on Sutures" (Vol. XLII, No. 3, p. 266) the corrected names of the authors are Ruth C. James and C. M. MacLeod.